Development of Recombinant Antibody Technology for Application in Plant Pathogen Diagnosis

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Bibliographic abstract

This thesis describes the applicability of the novel phage display technique in selecting single-chain Fv antibodies from combinatorial antibody libraries that are specific for plant-pathogens such as *Ralstonia solanacearum*, beet necrotic yellow vein virus and tomato spotted wilt virus. Several of the retrieved antibodies are applicable as coating and detection reagents in a double antibody sandwich ELISA or in immunofluorescence. This shows the potential of the phage display system in obtaining antibodies that are suitable for routine diagnosis without the use of laboratory animals.

Druk:
Ponsen & Looijen BV
Wageningen

STELLINGEN

1. Het selecteren van antilichamen met behulp van de "phage display" techniek vormt een goed en tevens diervriendelijk alternatief voor de hybridomatechniek.

   Dit proefschrift.

2. Gezien de miljoenen jaren evolutionaire ontwikkeling van het immuunsysteem is het naïef om te veronderstellen dat een combinatorial antibody library die geconstrueerd is uit B-lymfocyten van niet-geïmmuniseerde donoren inderdaad naïef is.

   Vaughan, T.J. et al., (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nature Biotechnology 14:309-314.

3. De "phage display" techniek is bij uitstek geschikt voor het signaal-transductie onderzoek.

4. Het baseren van een diagnostische toets op slechts één monoklonaal antilichaam is even riskant als het spelen van Russische roulette.

5. De afkorting SIP voor het faagselectiesyteem met behulp van "selectively infective particles", weerspiegelt ook de gemoedstoestand van de onderzoekers die dit systeem van de grond proberen te krijgen.

   Duenãs, M. and Borrebaeck, C.A.K. (1994) Clonal selection and amplification of phage displayed antibodies by linking antigen recognition and phage replication. Bio/Technology 12: 999-1002.

6. Het met succes tot expressie brengen van eiwitten in een heteroloog systeem berust meer op geluk dan op wijsheid.

   Levitt, M. et al., (1997) Protein folding: the endgame. Annual Review of Biochemistry 66: 549-579.

7. Waar cholera heerst groeien bananen, hetgeen deze dan ook tot een uitstekend 'adjuvants' maken voor het ontwikkelen van een oraal vaccin.

8. Het werken met kortlopende projecten is in de wetenschap een onderschatte vorm van geld- en kennisvernietiging.

9. Het verbod op het aanbrengen van reclame in proefschriften strookt niet met de commercialisering van de universiteiten.

10. Daar het tot nu toe niet gelukt is om het natuurlijke reservoir te vinden van diverse 'dierlijke virussen' is het, gezien de grote overeenkomst met 'plantenvirussen', aanbevelenswaardig om eens in het plantenrijk te gaan zoeken.

    Wijkamp, I. et al., (1993) Multiplication of tomato spotted wilt virus in its insect vector. Journal of General Virology 74:341-349 and Le Guenno, B. (1997) Haemorrhagic fevers and ecological pertubations. Archives of Virology 13:191-199.

Stellingen behorende bij het proefschrift:

Development of Recombinant Antibody Technology
for
Application in Plant Pathogen Diagnosis

Remko Griep, Wageningen, 17 maart 1999.

"...Against all odds..."

Contents

Chapter 1

General introduction

In the early days of agriculture crops were small, biodiversity was high and trade was limited. This situation gave plant pathogens only a limited chance to spread and to cause significant damage. However, as time past by, crops were covering larger areas. This, while the genetic homogeneity within the crops was increasing. In combination with the increasing trade, the application of monocultures in today's agriculture has resulted in the outbreak of many devastating plant diseases. The economic damage of diseases and pests, caused by viroids, viruses, bacteria, insects, fungi and plant-parasitic nematodes is gigantic. Indirect losses are difficult to assess because the presence of certain pathogens or plague organisms in productive soils discourages the cultivation of economically important plants.

Pathogens can be carried on, or in plant propagation material and a disease can become manifest when the crop starts to grow. Even when transmission rates are low, sufficient inoculum may be present for an epidemic outbreak. Certification of healthy plant propagation material is of major importance to avoid the spread of various diseases to regions where a particular plant-pathogen is absent. Moreover, the information obtained from general surveys, on the presence of plant-pathogens in or on crops or in soil, can be used for advisory systems with regard to crop rotation, cultivar selection, pesticide application, harvest dates, post harvest handling, etc.

Detection and identification

The earliest form of plant-pathogen diagnosis was based on the recognition of specific symptoms in crop plants. Because many pathogens do not produce specific symptoms in their hosts or are latently present, the development of alternative methods to confirm the identity of the causal agent is required. These assays should be robust, uncomplicated and fast. Transmission to indicator host plants, plating on selective media, microscopy, chromatography and electrophoresis often provide valuable information and are still widely used as methods for the identification and detection of plant-pathogens. However, they are laborious and it may take up to weeks before a result is obtained. Modern molecular techniques such as the polymerase chain reaction [58] and nucleic acid sequence based amplification [35] are fast and can be used as a reliable tool to detect various plant-pathogens [62,77]. Although both are sensitive methods, they are not suited yet for testing the large numbers that have to be tested annually (over 2 million samples in the Netherlands alone) for the certification of plant propagation material. In contrast, serological assays form a good compromise between robustness, sensitivity, specificity on one hand and ease and expense of application on the other hand.

Serological assays

The first report describing the use of serology for the identification of a plant-pathogenic bacterium was published in 1918 [31], when Jensen used an antiserum in an agglutination test showing that a strain of *Agrobacterium tumefaciens* from Denmark could be differentiated from a strain from the United States. During the 1930s, agglutination assays rapidly became popular for identification of plant-pathogenic bacteria and viruses, and remain so today. After the introduction of the agar double diffusion test by Ouchterlony [51], agar precipitin tests became widely used during the 1950s for identification of medically important bacteria. However, Ouchterlony double diffusion was not applied for identification of plant-pathogenic bacteria until 1960, when Lovrekovich and Klement [39] reported that species-specific antigens of *Pseudomonas tabaci* could be detected in Ouchterlony double diffusion tests but not in agglutination assays. Immunofluorescent (IF) staining was suggested in 1943 [52] for identification of bacteria in plants. However, no further interest was shown until 1965 when Morton [45] reported the superiority of direct IF over agglutination tests for rapid identification of the bacterium *Xanthomonas vesicatoria.* The development of the enzyme-linked immunosorbent assay (ELISA) by Van Weemen and co-workers [71] started a new era in direct detection of infectious agents. A few years after its development, this technique was also applied for the diagnosis of plant viruses [14].

The development of antisera against plant viruses has been very successful. In 1984, over 300 different virus species could be detected with specific immunoassays [69,70]. However, raising antisera specific for plant-parasitic nematode species, plant-pathogenic fungi and bacteria proved more difficult. Cross-reactions were generally observed with other nematode

species [78], saprophytic fungi [9] and closely related bacteria [44,68]. These problems can be ascribed to the variation in antibodies within a polyclonal antiserum.

Immune selection and the generation of polyclonal antibodies

The ability to respond to an apparently unlimited array of foreign antigens is one of the most remarkable features of the vertebrate immune system. Almost all antibody molecules contain a unique stretch of amino acids in their variable region (Fig. 1.1). This diversity is generated during B-lymphocyte differentiation (Fig. 1.2) in the bone marrow, where antibody encoding gene segments are randomly shuffled by a carefully regulated dynamic genetic system [27].

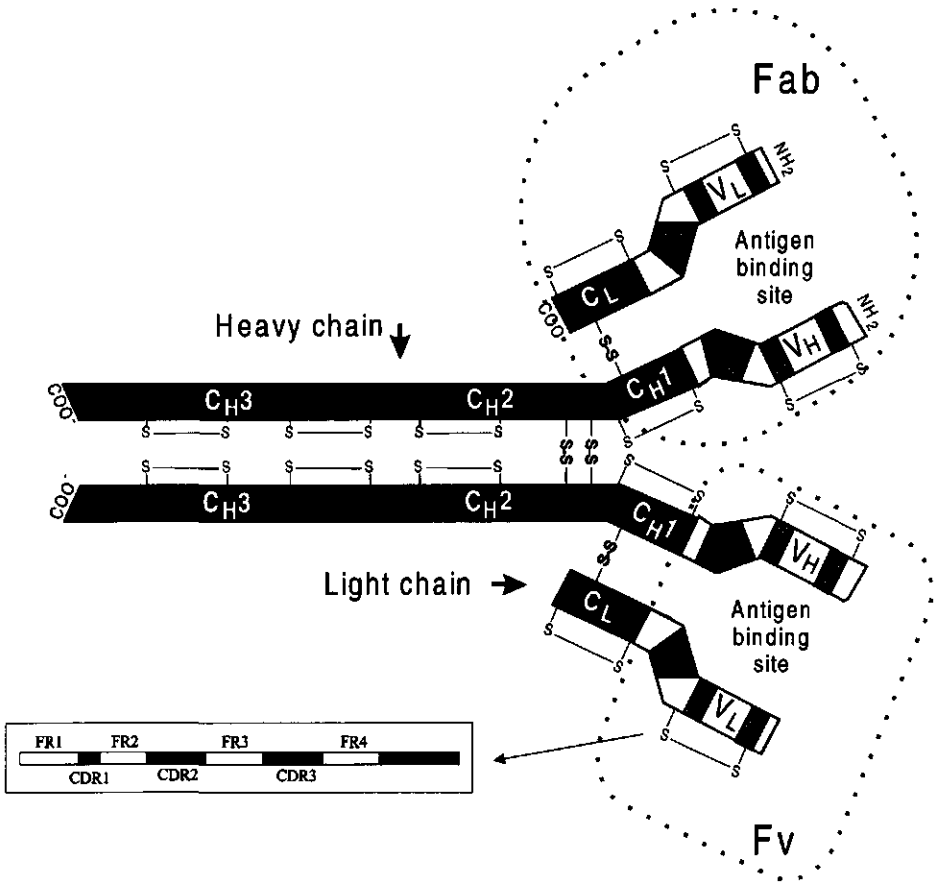

Figure 1.1. A model for the structure of a mouse antibody molecule, composed of two light and two heavy polypeptide chains. Two identical antigen-binding sites, formed by the variable regions of the heavy chain (V_H) and light chain (V_L), are located at one side of the antibody molecule. The constant regions of the light chain (C_L), the constant domains of the heavy chain (C_H1, C_H2, C_H3), interchain and intrachain disulfide bonds (S-S) are indicated. The Fv and Fab fragments of the antibody molecule are surrounded by dotted lines, whereas the organization of the variable domains in framework regions (FR) and complementarity determining regions (CDR) is shown in more detail within the box.

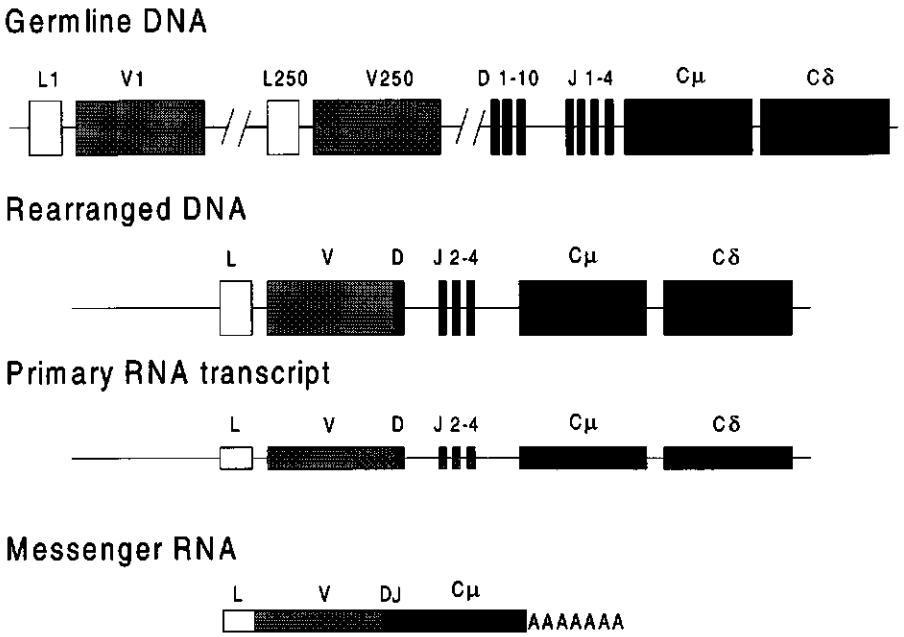

Figure 1.2. Sequence of rearrangements of immunoglobulin heavy chain genes involved in the formation of messenger RNA. The first rearrangement involves the recombination of V (variable), J (Joining) and in case of the heavy chain also D (Diversity) gene segments, so that rearranged VDJ genes are produced. In the primary RNA transcript the C region genes (Cμ and Cδ) remain separated from the VDJ complexes by introns. These introns are removed by RNA splicing to produce functional messenger RNAs for the μ heavy chain. Formation of light chain mRNA follows an essentially similar sequence, except that the D genes are absent (not shown).

Combinatorial joining of germline gene segments and two additional phenomena are active during this process: junctional flexibility and N-region nucleotide addition. The result is a mature immunocompetent B-lymphocyte, which contains a single functional DNA sequence encoding the heavy-chain variable-region (V_H) and a single DNA sequence encoding the light-chain variable-region (V_L). It is anticipated that the immune system is capable of generating more than 10^8 different antibody molecules (Table 1.1) without ever being exposed to foreign antigens. Upon antigenic stimulation this number is further increased (Table 1.1) by somatic hypermutation of the complementary determining regions in the V_L and V_H domains to at least 10^{10} different molecules [36].

Most plant-pathogens are complex mixtures and consist of multiple antigens containing two or more epitopes (antigenic determinants). These mixtures of antigens will cause clonal expansion of different T- and B-lymphocytes when injected into animals, (immunization). The resulting immune response is polyclonal and, in addition, polyepitopic [3]. As many epitopes are shared by related genera or species, cross-reactions can hardly be avoided when polyclonal antibodies are used in detection assays.

Table 1.1. The mechanisms generating the diversity in mouse antibodies. Combinatorial joining of germline antibody gene segments i.e., the variable regions of the light chain (V_L) and the heavy chain (V_H), the Diversity (D) and Joining (J) gene segments, together with junctional variability and N-region nucleotide addition enable an enormous variability within the encoded antibodies. Somatic mutation upon antigenic stimulation can further increase the variability.

Antibody heavy chain germline gene segments	Number of gene segments	Number of reading frames	Number of possibilities
V_H	200-300	1	± 250
D_H	10	3	30
J_H	4	3	12
Heavy-chain variation via combinatorial joining at V-D-J segments: 250 x 30 x 12 =			90,000
N nucleotide addition at V-D-J joints:			>90,000
Antibody light chain germline gene segments	Number of gene segments	Number of reading frames	Number of possibilities
V_L	100-300	1	± 250
J_L	4	3	12
Light chain variation via combinatorial joining at V-J gene segments : 250 x 12=			3,000
Total germline encoded variation in random association of any light chain with any heavy chain: >90,000 x 3,000=			>2.7 x 10^8
Somatic hypermutation in rearranged genes during the immune response			>10^{10}

Monoclonal antibodies

In contrast to polyclonal antibodies, monoclonal antibodies (MAbs) are defined by their origin from a single B-lymphocyte. Such antibodies are obtained by *in vitro* fusion of activated B-lymphocytes with a myeloma cell line [37]. The resulting hybrid cells (hybridomas) behave like tumor cells and undergo continuous proliferation. The antibodies secreted by a clone derived from a single hybridoma cell are monoclonal by definition. The main advantages of MAbs compared to conventional polyclonal antisera can be summarized as follows:

- Animal welfare. A reduction in the number of experimental animals can be achieved. There is no need for renewed immunizations after a successful cell fusion.
- Production of homogeneous and biochemically defined reagents. The standardization of MAb reagents will ensure that uniform results are obtained in all laboratories. In the past, the inherent variability of individual antisera available in only small quantities often led to inconsistent results. In contrast, MAbs enjoy a constant specificity and affinity and are theoretically available in unlimited amounts.
- Increased specificity. MAbs are specific for only a single epitope of the antigen and are therefore able to discriminate between isoenzymes and other closely related proteins [4].
- Selection of high affinity reagents. The screening procedure used to identify hybridoma clones secreting the required antibody allows the isolation of high affinity MAbs. Immunoassays carried out with such antibodies will be very sensitive with respect to antigen detection and concentration of antibody needed in the test.

Limitations in generating MAbs

Although, the number of MAbs directed against bacteria, fungi and nematodes is rapidly expanding [15,17,59,61,67], the many advantages should not obscure the fact that generation of MAbs against plant-pathogens is often laborious and troublesome. Upon immunization the immune response is triggered after recognition of foreign antigens by lymphocytes. As it is difficult to isolate pure plant viruses, a high percentage of the obtained hybridomas is often directed against the contaminating plant material that appears to be immunodominant [20]. For bacteria, fungi and plant-parasitic nematodes the choice of immunogen is difficult, as it is generally unknown which epitope is unique for a certain species. Moreover, another prerequisite is that this particular epitope has to be recognized by the immune system.

In addition, the hybridoma technique of Köhler and Milstein [37] does not satisfactory exploit the immune repertoire. While the antibody repertoire is estimated to consist of over 10^8 different antibodies (Table 1.1), only a few thousand antibodies are obtained, of which on average less than 1% are antigen-binding. With an optimized fusion protocol, e.g. electrofusion, a ten times higher fusion efficiency can be obtained [18,49], while antigenic *in vitro* stimulation or proliferation of affinity selected B-lymphocytes before fusion can yield an improved degree of antigen-specific hybridomas in some cases [66]. However, the problems of immunodominance and the enormous variability in epitopes present within the complex plant-pathogens also diminish the chance that a useful MAb will be obtained when these optimized procedures are applied.

Another major drawback of the hybridoma technique is that it is best suited for the generation of murine antibodies, while the main interest has always been in human antibodies for medical applications, e.g. tumor therapy and tumor imaging. In these applications murine MAbs offer no alternative, because they can give rise to an undesired immune response in humans [79]. Unfortunately, considerable difficulties in making human hybridomas exist [10], and moreover, they cannot be directed against human tumor antigens as these are usually regarded as self and do not elicit an immune response. Fortunately, it was realized that the problems associated with generating human B-lymphocytes could be circumvented through immortalizing antibody genes by recombinant DNA technology, rather than by immortalizing the cells themselves.

Antibody technology

The modular nature and conserved domain structure of antibodies [33] makes them attractive candidates for protein engineering and since 1984, recombinant DNA technology has revolutionized the production of MAbs. The initial recombinant antibodies were made by grafting the variable domains (V_L and V_H) of murine hybridomas on human constant domains [7,47]. Later, mouse complementary determining regions, the regions responsible for antigen binding, were put in a human framework [32,57,73] and expression of the chimaeric antibody genes in mammalian cells yielded humanized antibodies.

As yet, there are no reports of efficient expression of whole antibodies in bacteria, but subfragments (Fig. 1.1) such as Fabs (V_HC_H1 + V_LC_L) or Fvs (V_H + V_L) can be expressed well in *Escherichia coli* [5,55,63]. Spontaneous assembly of functional Fab fragments is observed when V_HC_H1 and V_LC_L domains are expressed as separate molecules. This association is in part driven by the hydrophobic interface between heavy and light chain residues. An interchain disulfide bond will stabilize assembled Fabs [22], but this is not a prerequisite for Fabs to be functional [26]. The key to expression of functional antibody fragments in *E. coli* appears to be export from the reducing environment of the cytoplasm into the oxidizing environment of the periplasm [56]. In contrast to Fab fragments, Fv fragments show a tendency to dissociate, particularly at low concentrations [21]. This can be overcome by connecting the C-terminus of one domain to the N-domain of the other with a flexible linker peptide [6,30] and expressing V_H and V_L domains as single-chain Fv (scFv) fusion proteins (Fig. 1.3).

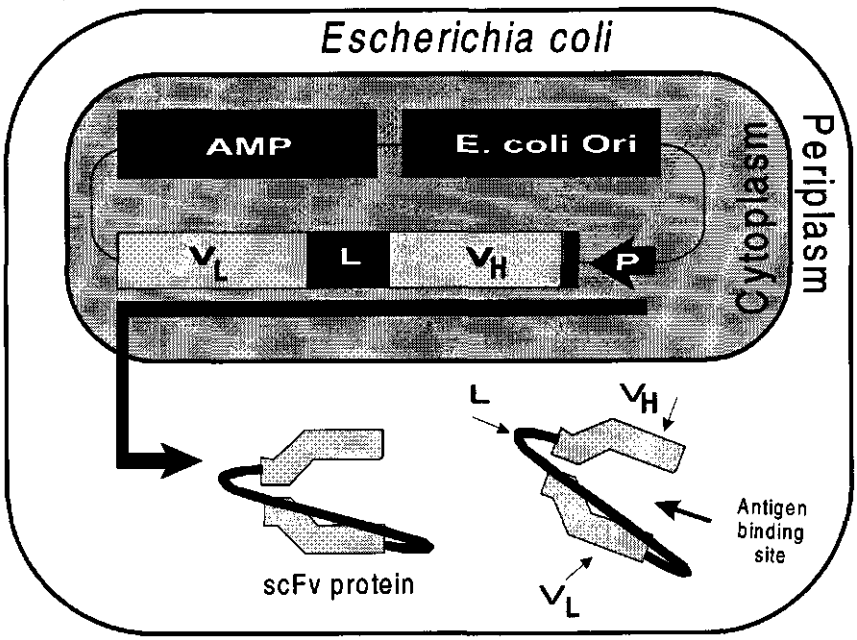

Figure 1.3. The variable domains V_H and V_L are joined by a linker peptide (L) and single-chain Fv (scFv) proteins are expressed and secreted into the periplasm of *Escherichia coli* after induction of the promoter (P) with a suitable inducer. In the scFv-encoding vector, the location of the signal peptide, the scFv gene, an ampicillin resistance gene (AMP) and an origin of replication (*E. coli* ori) are indicated.

Combinatorial antibody libraries of randomly assembled V_H and V_L genes

Initially, expression of antibody fragments in *E. coli* was achieved by cloning the unique heavy- and light-chain encoding cDNA sequences from hybridoma cells of known specificity. This cloning was simplified after the introduction of the polymerase chain reaction [58] that allowed specific amplification of known DNA sequences. Although the nucleotide sequences

encoding the complementary-determining regions are highly variable, the flanking regions have relatively conserved sequences [33]. In fact, the polymerase chain reaction provides a means to amplify and clone the V_H and V_L antibody repertoire encoding sequences directly from B-lymphocytes of immunized mice [38,50,60,75], thereby omitting the need for the generation of hybridomas. The resulting libraries were combinatorial as the amplified V_H and V_L genes were randomly recombined irrespective of their original pairing. Within biased combinatorial libraries (derived from activated B-lymphocytes of hyper-immunized mice) the number of antigen-specific V genes is high, at best <1/500 [46] and more usually <1/5000 [11,54]. These libraries can therefore be relatively small (10^5-10^6) to regain original V_H and V_L pairings.

While it is difficult and time consuming to identify MAbs produced by hybridomas, the ease by which *E. coli* can be manipulated reduces the time required for antibody production and characterization. Bacterial expression of diverse combinatorial antibody libraries allows screening of a larger population of antibody fragments (50,000 per filter) than could be tested previously using conventional methods [28,64]. However, this screening process was still very laborious, especially in view of the huge number of clones generated using combinatorial libraries. This difficulty in isolation of fragments with binding activity has been largely overcome by the development of techniques for the display of antibody fragments on the surface of filamentous bacteriophage.

Mimicking the immune system by display of antibody fragments on bacteriophage

Filamentous bacteriophage were first used to display small peptides on the minor coat protein pIII of bacteriophage Fd, and very rare peptides could be isolated [65] through sequential cycles of phage growth and selection by binding to antibody. When it appeared that larger proteins, among which scFv-antibodies [43] or Fab fragments [25], could also be expressed functionally on the surface of bacteriophage (Fig. 1.4), a powerful selection system was created to obtain specific MAbs from combinatorial phage-antibody libraries [13].

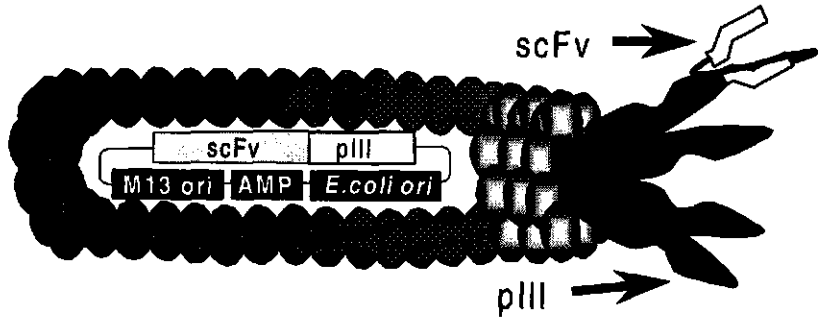

Figure 1.4. Expression of a scFv-antibody fragment on bacteriophage through genetic fusion of a scFv gene with the gene of minor coat protein pIII. In the scFv-encoding phagemid vector, the scFv gene, an ampicillin resistance gene (AMP), a packaging signal (M13 ori) and an origin of replication (*E. coli* ori) are indicated. During phage assembly the scFv-pIII encoding phagemid is packaged into the phage particle and the scFv-pIII fusion protein is expressed on the phage surface. Consequently, the phenotype and genotype are linked.

In the immune system the B-lymphocytes represent self-replicating packages. They contain the antibody genes that encode the antibody displayed at their surface. Filamentous bacteriophage expressing functional antibody fragments on their surface, thus, mimic the B-lymphocyte by linking genotype and phenotype.

The phage display system (Fig. 1.5) allows direct selection of antibodies with rare specificities from combinatorial phage display antibody libraries through successive rounds of phage growth and selection for antigen binding. For instance, Bradbury and co-workers [8] showed the feasibility of this technique by selecting antigen binding phages from a pool of non-binding phages, even at a ratio of one binding in 10^9 irrelevant phages.

Presently a variety of formats for antibody phage display exist. Antibody fragments can be cloned directly into the genomes of filamentous bacteriophage or, alternatively, phagemid vectors can be used. These plasmid-based vectors have sequences derived from the intergenic regions of filamentous phage, which enable them to replicate as a single stranded DNA in *E. coli*. These phagemids are packaged into filamentous phage particles (rescued) when cells harboring them are co-infected with helper phage such as M13K07 [74], that provide all the phage proteins but due to a defective origin they are themselves poorly packaged in competition with the phagemids.

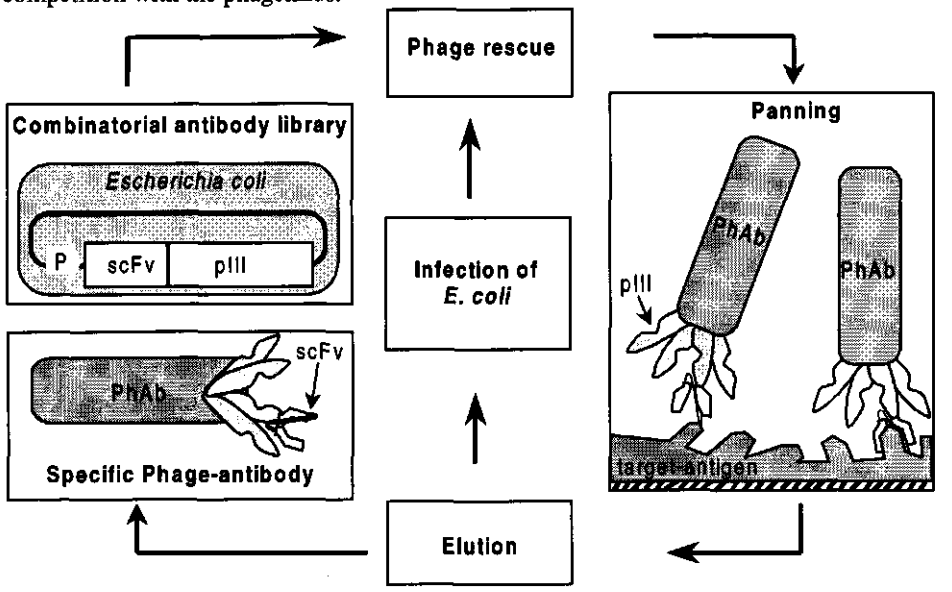

Figure 1.5. Schematic representation of the selection of antigen-specific single-chain antibody fragments (scFvs) from a combinatorial antibody library using phage display. Helper phage, which contain the entire phage genome but lack an efficient packaging signal, are used to 'rescue' phagemids from a combinatorial library. When both helper phage and phagemid are present within the same bacterium, phage-antibodies (PhAbs) are assembled that carry fusion proteins of scFv and minor coat protein pIII on their surface and contain the scFv-encoding phagemid vector. In order to select for antigen specificity, PhAbs rescued from a combinatorial antibody library are allowed to bind to immobilized antigen (Panning). Washing removes the PhAbs that lack affinity for the antigen. Bound PhAbs are eluted, and the selected PhAbs are enriched by sequential rounds of panning until the desired affinity is obtained.

The number of antibodies, displayed on each phage particle, can be varied to a great extend. Antibody fragments can be genetically fused to the gene encoding the minor coat protein pIII (3-5 copies per particle), and be displayed at 3 (possibly 5) copies per bacteriophage [1,19,25,43]. The major coat protein pVIII (2700 copies per particle) can also be used to display antibodies on phage [12,25,29,34], but these phages are not viable unless wild-type pVIII coat protein is provided by helper phage. The use of the phagemid/helper phage system leads, through competition for wild-type coat proteins to fewer copies of the antibody fusion being displayed. With helper phage providing wild-type pVIII and pIII coat proteins, approximately 24 antibody fragments are incorporated per phage if antibody-pVIII encoding phagemids are used [34], whereas less than one antibody is incorporated per phage for antibody-pIII fusions. Because avidity effects are avoided, fusions to pIII coat proteins have to date been preferred over pVIII for antibody display and were used to select high affinity antibodies from combinatorial antibody phage display libraries using a wide variety of selection strategies.

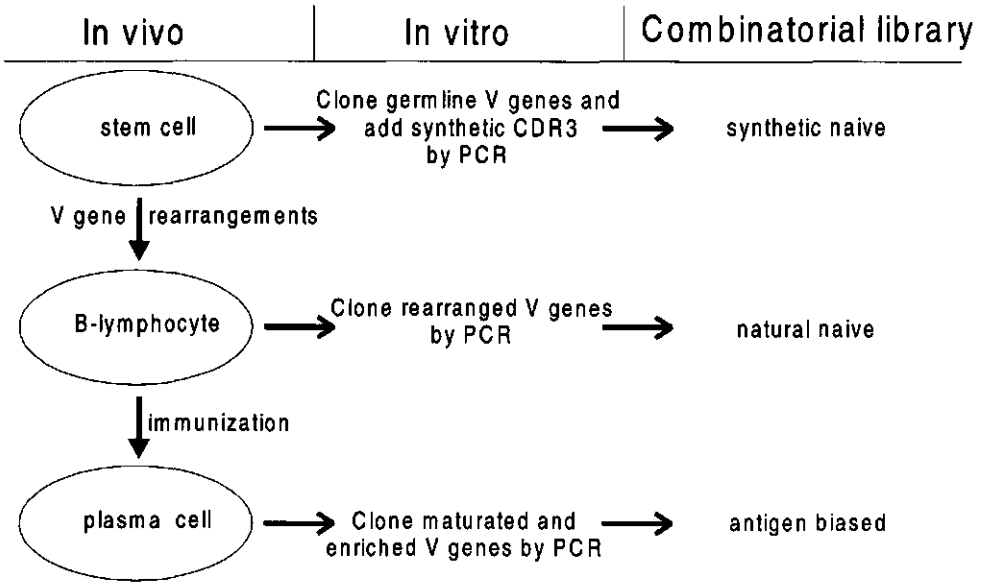

Figure 1.6. Antibody production *in vivo* and *in vitro* and the type of combinatorial library that can be constructed using the PCR amplified V genes derived from stem cells, B-lymphocytes or plasma cells (activated B-lymphocytes).

Bypassing immunization

The major disadvantage of biased combinatorial antibody libraries, assembled from B-lymphocytes of immunized sources, is that for each antigen a new library has to be constructed. Moreover, immunization of humans for this purpose is not ethical and self-antigens are not suited to elicit an immune response. However, it is possible to bypass immunization and to construct naive combinatorial phage display antibody libraries using V genes of non-immunized sources (Fig. 1.6). The probability of isolating high affinity

antibodies towards every imaginary antigen from such a repertoire is dependent on the diversity of the library [53]. Therefore, these naive antibody phage display libraries have to be as large as possible. Naive human combinatorial antibody phage display libraries have been constructed that are comparable to the natural immune repertoire with a diversity of approximately 10^8 scFv-antibodies [41] or even more diverse, with 10^{10} scFv-antibodies [72]. Using an *in vivo* recombination technique, which applied the Cre-enzyme of phage P1 [76], construction of a naive combinatorial Fab phage display library with a diversity of 10^{12} was achieved [23].

Alternatives for the use of rearranged V repertoires from a non-immunized source are cloned human germline V_H and V_L sequences. Using these genes, amplified with a specific 5' end and a synthetic CDR3 primer (Fig. 1.6), large (>10^8) synthetic combinatorial antibody libraries [2,26,48] were constructed. In addition, a synthetic Fab phage display library (10^8) was constructed in which the natural structure of the CDR3 was retained [16].

Selection of specific antibodies from combinatorial antibody phage display libraries

Naive and synthetic combinatorial antibody phage display libraries encode antibodies of considerable diversity and can theoretically be used to select antibodies against many different antigens. In addition, the phage display system may also prove to be a more universal method to obtain specific MAbs, since the antibody specificity is not biased towards immunodominant epitopes as immunization procedures are omitted. Phages, encoding displayed antibody fragments, can be affinity selected from combinatorial antibody phage display libraries for binding to a particular antigen by passing the phage-antibodies over immobilized antigen (panning). Several formats have been used, such as immobilized antigen on a column matrix [43], antigen-coated plastic tubes or dishes [41], biotinylated antigen in solution followed by capture on streptavidin coated beads [24] and whole cells [16,42].

Phages that are bound to antigen are retained on the surface. Non-binding phage-antibodies are removed by washing. Bound phage-antibodies can be eluted by aspecific elution using acid [1] or alkaline buffers [40] or more specifically using competition with soluble antigen [13], proteolysis of spacers located between phage and antibody or reduction of disulfide bonds in biotinylated antigens. The selected (eluted) phages are used to infect *E. coli* and can be applied in several sequential rounds of phage growth and selection, allowing even rare phage-antibodies to be isolated.

Screening of the selected antibodies

The success of the panning against an antigen can be assessed, using a phage-ELISA for screening the specificity of the selected phage-antibodies (Fig. 1.7). Alternatively, one of the more useful features in phage display vectors, the presence of an amber stop-codon at the junction between the antibody and the coat protein, can be used for facile switching between fused and soluble antibodies (Fig. 1.7). Because most recombinant antibodies have a peptide tag fused to the C-terminus, the reaction towards antigen can also be determined in ELISA.

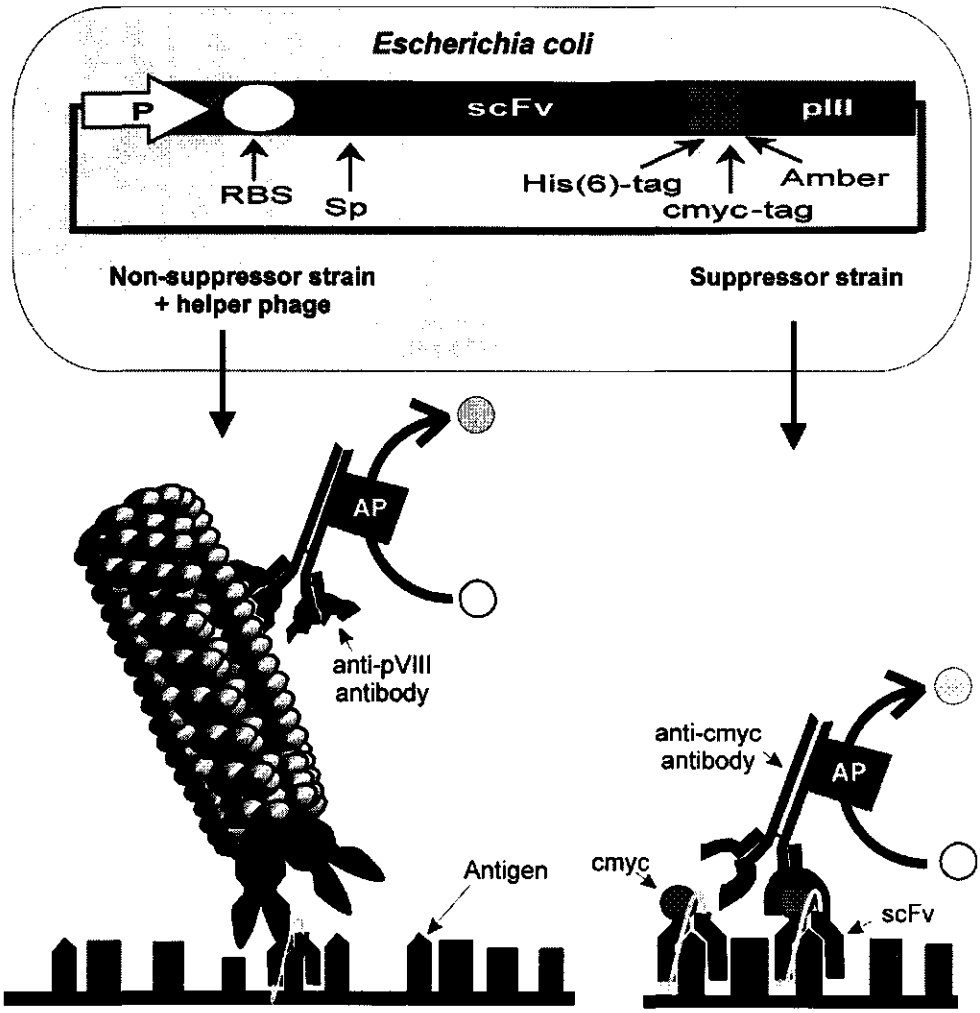

Figure 1.7. The application of an amber codon allows facile switching from production of phage-antibodies to soluble scFvs by changing the non-suppressor strain (wherein translation proceeds at the amber codon) to a suppressor strain (wherein translation halts at the amber-codon). In the scFv-encoding vector the location of a ribosomal binding site (RBS), a signal peptide (Sp), a scFv, a His(6)-tag, a cmyc-tag, an amber codon and the pIII gene are indicated. The produced phage-antibodies can be applied in phage-ELISA (lower left) and the soluble scFvs in cmyc-ELISA (lower right). Bound phage-antibodies or scFv antibodies can be detected with alkaline phosphatase (AP) labeled anti-phage antibodies or anti-cmyc antibodies, respectively. Visualization of the reaction can be achieved by addition of a colorless substrate that is modified into a colored compound by AP.

Objective of this thesis

Many MAbs against a broad variety of antigens have been isolated from naive and synthetic combinatorial antibody libraries with the aid of the phage display technique [23,48,72]. Therefore, it appears that this technique offers features that can not be achieved using the hybridoma technique, i.e. high variability in the antibody repertoire and, as no laboratory animals are required for immunization purposes, there is no bias towards immunodominant epitopes. Moreover, production of recombinant antibodies in bacteria is fast and relatively cheap. Based on these features, the phage display technique might be well equipped to select plant-pathogen specific MAbs from combinatorial antibody libraries.

The objective of this thesis is to show that the phage display technique is indeed applicable for selection of specific recombinant MAbs that can be used in reliable and sensitive immunoassays for the detection of pathogens in or on plant material. Therefore, the phage display technique was used to select recombinant scFv-antibodies against several plant-pathogens of which it is known that specific hybridoma derived MAbs can be selected only with great difficulty.

In the second chapter we describe the selection of *Ralstonia solanacearum*-specific MAbs from a naive combinatorial scFv library with the aid of the phage display technique. The selected scFv-antibodies were screened for their specificity for *R. solanacearum* by testing them against several closely related as well as a panel of saprophytic bacteria.

In Chapter 3 is described that several beet necrotic yellow vein virus-specific monoclonal scFv-antibodies were selected from a synthetic combinatorial scFv library. Initially, these scFv-antibodies reacted only when they were displayed on phage, but not when they were expressed as soluble protein. An improvement in expression was achieved by expression of the scFvs as fusion protein with alkaline phophatase.

It was realized that the inability to express scFvs as soluble proteins was a general problem. Chapter 4 describes the optimization of expression and screening procedures, starting with the observation (Chapter 3) that several of the selected scFv could be expressed as scFv-alkaline phosphatase fusion protein but not as soluble scFvs.

In the fifth chapter, the optimized screening and expression procedures are applied to select the recombinant antibodies directed against tomato spotted wilt virus. This finally resulted in the development of a sensitive ELISA that is fully based on recombinant antibodies.

In Chapter 6, it is shown that besides genetic fusion of scFv-antibodies to enzymes like alkaline phosphatase, fluorescent proteins can also be used as a versatile fusion partner. The obtained fluorescent antibodies (fluobodies) can be directly used in immunofluorescent cell staining and flow cytometry.

In the summary and concluding remarks section (Chapter 7), the use of the phage display system and naive combinatorial antibody libraries is discussed for the selection of MAbs against plant-pathogens. Recent data, obtained from testing laboratories, will be used to show that several of the selected recombinant antibodies can be used in routine diagnosis.

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Chapter 2

Development of specific recombinant monoclonal antibodies against the lipopolysaccharide of *Ralstonia solanacearum* race 3

ABSTRACT

Recombinant single-chain antibodies (scFvs) against the lipopolysaccharide of *Ralstonia solanacearum* (biovar 2, race 3) were successfully selected by phage display from a large combinatorial antibody library. Characterization with regard to cross-reaction and use in routine immunoassays showed that the selected antibodies had improved characteristics when compared to the polyclonal antiserum which is currently used for 'brown rot' diagnosis of potato in the Netherlands. The isolated monoclonal scFv antibodies reacted both in ELISA and in immunofluorescence cell staining with all race 3 strains tested but only with some strains belonging to other races. Furthermore, only a few cross-reactions with saprophytic bacteria that cross-reacted with polyclonal antisera were observed. One of the recombinant antibodies detected as few as 5 x 10^3 bacteria in potato tuber extracts in ELISA. Therefore, this antibody is potentially useful for detection of *P. solanacearum* race 3.

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INTRODUCTION

Ralstonia solanacearum is the causal organism of bacterial wilt and can infect over 450 different plant species, including many economically important crops [7]. The genus *R. solanacearum* (formerly *Pseudomonas solanacearum*) can be classified into 3 races (designated according to host range specificity) or grouped into 5 biovars on the basis of utilization of disaccharides and hexose alcohols. Race 3, nearly synonymous with biovar 2, forms a homogeneous group [26] and its host range is restricted to potato, eggplant and tomato. Race 3 is more adapted to temperate regions and is responsible for recent outbreaks of potato brown rot disease in different countries in Europe [27].

Quarantine measures have been taken in many countries to minimize the risk of introduction and spreading of the potato brown rot pathogen. Serological techniques, such as ELISA, immunofluorescence cell-staining (IF) and immunofluorescence colony staining (IFC), are commonly used to monitor the presence of the pathogen in potatoes and in the environment. In general, they are considered to be a good compromise between sensitivity and specificity of detection, and ease and expense of application [5, 22, 25].

So far, only polyclonal antibodies (PAbs) against whole or glutaraldehyde-fixed cells of *R. solanacearum* race 3 have been used in certification and risk assessment programs [5, 11]. However, the occurrence of false-positive reactions due to the presence of cross-reacting saprophytic bacteria in the extracts is considered a serious drawback to the use of PAbs. In IF cell staining, up to 3% false-positive reactions were recorded [11] while in ELISA the frequency of false-positive reactions can sometimes exceed this level (Elphinstone, personal communication). Consequently, positive reactions need to be followed by confirmation assays, for which often tedious and time-consuming bioassays have to be used.

In contrast to PAbs, hybridoma-derived monoclonal antibodies (MAbs) recognize only one epitope and thus a higher level of specificity is feasible. Attempts to produce MAbs against *R. solanacearum* were successful [1, 2, 8, 22]. However, the derived MAbs were not applicable for brown rot diagnosis in potatoes. The species-specific IgM MAb Ps1, raised by Alvarez *et al* [1, 2], reacted with extracellular polysaccharide-I in a very sensitive immunoassay [17] but failed to react with afluidal mutants [2] which can still be pathogenic [31], despite the phenotype conversion. Moreover, MAb Ps1 is not applicable for IFC in agarose plates for its large molecular mass of 900 kDa reduces gel diffusion and slows down staining and washing procedures. The MAbs developed by He [8], reacted with race 1 and 2, but not with race 3 strains [8]. Strain-specific MAbs that do not cross-react with the most closely related species (*R. picketti*, *R. syzygii*, *Burkholderia cepacia* or the banana blood disease bacterium) were developed successfully by Robinson *et al* [22]. However, these MAbs were not useful for certification of potato propagation material, as they did not react with 11 of 23 *R. solanacearum* strains tested, including two race 3 strains. In addition, they did not react in IF-techniques and a 100-fold decrease in sensitivity was observed in ELISA when PAbs were replaced by MAbs.

The difficulties mentioned above, in developing a *R. solanacearum* race 3 specific MAb which is suited for potato brown rot diagnosis using ELISA, IF and IFC techniques, might be caused by inefficient exploitation of the immune repertoire in the hybridoma technique. After all, whereas the available (mouse) antibody repertoire is estimated to include over 10^8 different antibodies, only a few thousand different hybridoma clones are obtained, of which on average less than 1% produce antigen binding antibodies. Thus, the probability of selecting MAbs of sufficient specificity and affinity for use in a diagnostic assay is low.

These diversity and efficiency problems were solved by recent advances in molecular immunology (Chapter 1). Forced cloning of antibody variable heavy (V_H) and light (V_L) chain genes by RT-PCR [20, 24] allowed amplification of the DNA encoding the antibody repertoire. Coupling V_H and V_L domains with a flexible peptide linker [10] enabled expression of variable antibody fragments (Fv) in Escherichia coli as single-chain Fv (scFv) molecules. Cloning of a pool of scFv encoding genes, in which the V_H and V_L domains were randomly combined, allowed the generation of large combinatorial antibody libraries. Today combinatorial libraries of over 10^{10} different antibodies have been constructed [6, 30]. This, in combination with the display of functional antigen binding fragments on the tips of filamentous phage created a powerful system to select specific MAbs [6,9,16,30] without the need for immunization i.e. without the use of experimental animals.

In this study, the versatility of the phage display system was challenged for the selection of *R. solanacearum* specific MAbs. To achieve this, phages derived from a large naive human combinatorial antibody library [30] were panned against purified lipopolysaccharides (LPS) of *R. solanacearum* and the expressed scFv-antibodies were characterized by ELISA and IF.

MATERIALS AND METHODS

Bacterial strains and growth conditions

E. coli strains used for the isolation of recombinant antibodies, were TG1 (K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*D5/F'*tra*D36, *pro*A^+B^+, *lacI*^{q}, *lacZ*ΔM15) for selection of specific phage-antibodies and HB2151 (K12, *ara*, Δ*lac-pro*), *thi*/F'/*pro*A^+B^+, *lacI*^{q}ZΔM15) for production of soluble scFv-antibody fragments.

Strains of *R. solanacearum* and strains of cross-reacting bacteria were preserved at -80°C on beads (Protect Bacterial Preservers, Biotrading Benelux, Wilnis, NL) in 15% glycerine, 8 mg/ml LabLemco broth (Oxoid, CM15). For each experiment, bacteria were grown for 48 to 72 h at 27°C on plates of Trypticase Soy Broth (30 g/l; BBL), and prior to use transferred to slopes of growth factor agar (2.3 mM K_2HPO_4, 0.2 mM $MgSO_4.7H_2O$, 1.7 mM NaCl, 4.3 mM $NH_4H_2PO_4$, 5 mM glucose, 0.5% (w/v) yeast and 1.5 % (w/v) agar, pH 7.2) and grown for 24 h at 27°C. Characterization of cross-reacting bacteria by fatty acid analysis, using the Microbial Identification System (Microbial ID, Newark, Delaware, USA), was kindly done by Dr. J.G. Elphinstone [Central Science Laboratory (CSL), York, UK].

Purification of lipopolysaccharides

The purification of lipopolysaccharides (LPS) from *R. solanacearum* (strain 1609: race 3, biovar 2) was performed according to De Weger *et al* [4].

Panning procedure

Selection of phage-antibodies (phages expressing functional scFvs on their surface) from the human combinatorial antibody library was performed according to Vaughan *et al* [30] with a few modifications. Immunosorbent tubes (Maxi-sorb, Nunc) were coated with 500 µg LPS (125 µg/ml in 50 mM $NaHCO_3$, pH 9.8) for 18 h at 4°C. The tubes were washed twice with PBS and blocked with PBM-2% (PBS containing 2% skimmed milk powder) for 30 min at room temperature. Simultaneously, 2 ml of a stock derived from the Vaughan library [30], containing 2.5 x 10^{13} phages displaying scFv antibodies (PhAbs), was mixed with 2 ml of PBM-4% and preincubated for 30 min. After removing the blocking solution from the tubes and washing with PBS, 4 ml of PhAbs was added to the tubes. PhAbs were allowed to bind to LPS for 30 min on a roller bench and for another 90 min without rotation. Free PhAbs were removed by washing the tubes 10 times with PBS containing 0.1% Tween-20 and for another 10 times with PBS to remove the detergent. Bound PhAbs were eluted by adding 1 ml 0.1 M triethylamine (TEA) and subsequent incubation for 10 min on a roller bench. After collecting the TEA eluent, the pH was neutralized by addition of 0.5 ml 1 M Tris/HCl pH 7.4. One ml of the eluted PhAbs was used to infect 5 ml of *E. coli* TG1 bacteria (or HB2151 in the final panning round), which were freshly grown to an OD_{600} of 0.5 in 2 x tryptone yeast broth (2TY, [23]). After infection (30 min, water bath at 37°C without shaking to allow optimal infection), the *E. coli* cells were pelleted (3,000 x g, 10 min) and subsequently resuspended in 1 ml of 2TY broth containing 100 µg/ml ampicillin and 2% glucose (2TYB-AMP-2%Glu). To establish the number of eluted PhAbs, 50 µl was taken from this suspension and serial dilutions were plated on 2TY agar plates containing 100 µg/ml ampicillin and 2% glucose (2TYA-AMP-2%Glu). The remaining 950 µl was plated separately on 225 X 225 mm 2TYA-AMP-2%Glu plates, and the bacteria were grown for 18 h at 30°C.

This procedure was carried out four times in succession, using PhAbs prepared as described below for the second, third and fourth panning round. To increase the specificity and to reduce the background the stringency was increased after the second panning round. The amount of antigen that was used for coating was then decreased from 125 µg/ml to 10 µg/ml in the third and to 1 µg/ml in the final panning round. Simultaneously, the stringency was further enhanced by increasing the number of washings from 20 times after the second panning round to 40 times in the subsequent rounds of selection.

Preparation of phage-antibodies for subsequent panning rounds

The bacteria, derived from a previous panning round, were scraped from the plate (225 x 225 mm) and resuspended in 15 ml of 2TYB-AMP-2%Glu. From these bacteria 14.5 ml was used to prepare a freezer stock [22] and 500 µl was used to inoculate 50 ml of 2TYB-AMP-1%Glu and the bacteria were grown at 37°C with shaking (250 rpm). When an OD_{600} of 0.5 was reached, 10^{11} helper phages (M13K07, Pharmacia, Uppsala, Sweden) were added (multiplicity of infection 20). The 15 ml tube containing the mixture was put in a water bath at 37°C, without shaking to allow for optimal infection. After 30 min the bacteria were pelleted (2,100 x g, 10 min) and resuspended in 25 ml 2TYB-AMP and 25 µg/ml kanamycin. The bacteria were transferred to a 250 ml Erlenmeyer flask, and grown for 18 h at 30°C with shaking (250 rpm). The 25 ml overnight culture was harvested and the bacteria were removed by centrifugation (2,100 x g, 20 min). The PhAbs in the supernatant were precipitated by adding 5 ml of 20 % PEG-6000, 2.5 M NaCl and thoroughly mixed for 1 h at 4°C. The precipitated PhAbs were pelleted (2,100 g, 20 min) and resuspended in 1 ml of sterile PBS. Usually 5 x 10^{12} PhAbs/ml were produced, as assayed by infection of strain TG1 and plating for ampicillin resistance. The derived PhAbs were stored at 4°C until use in the next panning round or in phage-ELISA.

***Mva*I-fingerprinting**

To analyze the diversity of the selected PhAbs, restriction fragment length polymorphism (RFLP) fingerprinting was performed on PCR amplified scFv DNA. Single colonies were picked and grown for 4 h in 2TYB-Amp-2%Glu. From these suspensions, 2 µl was taken and added to a 48 µl PCR mix containing 2.5 µM dNTPs; 0.25 U Super Taq DNA polymerase (HT Biotechnology, Cambridge, UK); 10 µM forward (5'-AGG AAA CAG CTA TGA CCA TGA TTA CGC CAA G-3') and 10 µM reverse (5'-GCC CAA TAG GAA CCC ATG TAC CGT AAC ACT G-3') primers; 2 mM $MgCl_2$ and 50 mMTris/HCl, pH 8. In a thermal cycler (Perkin Elmer), 25 cycles (1 min 94°C; 2 min 72°C) were performed. From the PCR mix, 20 µl was added to 37.5 µl H_2O and 6.5 µl of buffer H (Boehringer, Mannheim, Germany). After mixing, 10 U *Mva*I (Boehringer) was added and the mixture was incubated for 18 h at 37 °C. The *Mva*I digestion patterns were analyzed on a 3% FMC Metaphor agarose gel (Epicentre Technologies, Madison, USA).

Production and Purification of scFvs

HB2151 bacteria were cultured according to Kerschbaumer *et al* [12] and pelleted. After incubation (5 min, 0°C) of the bacteria with 1/20 volume (referring to the original culture size) 50 mM Tris/HCl, pH 8 buffer containing 30% sucrose and 1 mM EDTA, the scFvs were extracted (45 min, 0°C) from the periplasm with 1/20 volume (referring to the original culture size) of 5 mM $MgSO_4$. Secreted scFvs were purified from the periplasmic fraction using immobilized metal affinity chromatography (IMAC) according to Lindner *et al* [15].

Routine protocol for the extraction of potato tubers

The extraction of potatoes was carried out according to the legislation of the European Community (97/647/EG). Briefly, maceration buffer (4.26 g Na_2HPO_4 en 2.72 g KH_2PO_4 (50 mM PO_4) per liter of distilled water, pH 7.0) was added till 200 heel ends were fully submerged. The sample was incubated during 18 h at 6°C while shaking (75 rpm) and filtrated through a 40 to100 µm filter. Filtrate was collected after washing the filter with maceration buffer and centrifuged during 15 min at 10,000 g at 6°C. The pellet was resuspended in 1 ml of 10 mM phosphate buffer (2.7 g/l of $Na_2HPO_4.12H_2O$ and 0.4 g/l of $NaH_2PO_4.2H_2O$, pH 7.2). Glycerin was added to a final concentration of 15% and samples were stored at -70°C.

ELISA

A phage-ELISA was used to assess the specificity of the phage-antibodies which were derived from the for panning rounds, according to standard methods [3, 28], in which the plates were washed four times with PBST (PBS containing 0.1% Tween-20) between each incubation step. Briefly, a 96-well microtiter plate was coated with LPS (2 µg/ml in 0.1 M $NaCO_3$), for 2 h at 37°C at 100 µl/well. After blocking with PBSTM-5% (PBS containing 0.1% Tween-20 and 5% skimmed milk powder) at 200 µl/well, the plates were incubated for 1 h with PhAbs in PBSTM-1% at 100 µl/well. After that, the plates were incubated at 100 µl/well with anti-M13 monoclonal antibodies (MAb 12E4, diluted to 5 µg/ml in PBSTM-1%) and finally with rat anti-mouse PAbs conjugated to alkaline phophatase (RaAM-AP, Jackson Immuno Research Laboratories Inc., Westgrove, PA), diluted 1:2,000 in PBSTM-1%. The reaction with LPS was visualized by incubation with *p*-nitrophenyl phosphate substrate (p-NPP).

An indirect ELISA was used to assess the specificity of the recombinant scFv-antibodies, according to standard methods [3, 28], in which the plates were washed three times with PBST (PBS containing 0.1% Tween-20) between each incubation step. Briefly, a 96-well microtiter plate was coated, either with LPS (2 µg/ml in 0.1 M $NaCO_3$) or with bacteria (OD_{600} of 0.1 in 0.1 M $NaCO_3$), for 2 h at 37°C at 100 µl/well. After blocking with PBSTM-5% at 200 µl/well, the plates were incubated for 1 h with PAbs PcA-9523 (a polyclonal antiserum which was produced against whole cells of *R. solanacearum* race 3 strain 1609, diluted 1:2000 in PBSTM-1%; PAb-ELISA) or with scFvs (cMyc-ELISA) diluted in PBSTM-1% at 100 µl/well. After that, the plates were incubated at 100 µl/well, with goat anti-rabbit PAbs conjugated to alkaline phosphatase (GaR-AP, Sigma, 1:2,000; PAb-ELISA) or anti-cMyc monoclonal antibodies (MAb 9E10 [18], diluted to 1 µg/ ml in PBSTM-1%; cMyc-ELISA [9] and finally with RaAM-AP (Jackson), diluted 1:2,000 in PBSTM-1%. The reaction with LPS was visualized by incubation with p-NPP.

The detection limits of the scFv-antibodies and polyclonal antiserum PcA-9523 for *R. solanacearum* were determined with an indirect double antibody sandwich ELISA, in which the plates were washed three times with PBST between each subsequent incubation step. Briefly, ELISA plates (Greiner, intermediate binding capacity: cat. no. 655001) were coated

with PAbs PcA-9523 (diluted 1:2,000 in 0.1 M $NaCO_3$) at 100 µl/well for 2 h at 37°C. After blocking with PBSTM-5% for 30 min at 200 µl/well, the plates were incubated with ten-fold serial dilutions of *R. solanacearum* bacteria (diluted in either PBS or in potato tuber extract) at 100 µl/well for 2 h at 37°C. Subsequently, the plates were incubated with either PcA-9523-AP conjugate (diluted 1:2,000 in PBM) or with scFv anti-LPS 12 (diluted to 5 µg/ml in PBM-2%) at 100 µl/well for 1 h at 37°C. This was followed by subsequent incubations with anti-cMyc MAb 9E10 (1 µg/ml in PBM-2%) and RaAM-AP (1:2,000 in PBM-2%) at 100 µl/well for 1 h. The reactions were visualized by incubation with either p-NPP substrate or with fluoresceindihosphate (FDP) substrate. Optical densities at 405 nm were measured with an Anthos ELISA-reader and fluorescence was measured at 510 nm with a fluorometer (Perkin Elmer, 7700).

Western blotting

Purified LPS (0.5 µg/lane) was separated by SDS-PAGE and transferred to nitrocellulose membranes as described by Van der Wolf *et al* [29]. Reactive groups on the membrane were blocked with PBMT-5%. The reactivity of the scFv towards LPS was shown by subsequent incubations of the blot with anti-LPS scFv, anti-cMyc MAb 9E10 (10 µg/ml in PBM-2%), RaAM-AP (1:2,000 in PBM-2%) and BCIP substrate.

Immunofluorescence cell staining

IF was performed according to Van der Wolf *et al* [29]. For IF cell-staining the bacteria were coated on microscope slides and subsequently incubated with anti-LPS scFv (12,5 to125 µg/ml PBS) and anti-cMyc MAb 9E10 (30 µg/ml PBS), which was conjugated with fluorescein isothiocyanate (FITC).

RESULTS

Selection of LPS binding clones from a combinatorial antibody library

From the first panning round in which 2.5 x 10^{13} PhAbs (expressing 1.4 x 10^{10} different scFvs) were applied to the LPS coated immunosorbent tube, approximately 1.5 x 10^{6} PhAbs were recovered (Fig. 2.1A). In the subsequent rounds of selection, enrichment factors of 100 to 1000-fold were obtained, as the stringency during the selection was gradually increased. Increasing signals in phage-ELISA (Fig. 2.1B) paralleled the increased efficiencies of recovery during panning. This result indicates that phages binding to LPS were enriched during the selection procedure.

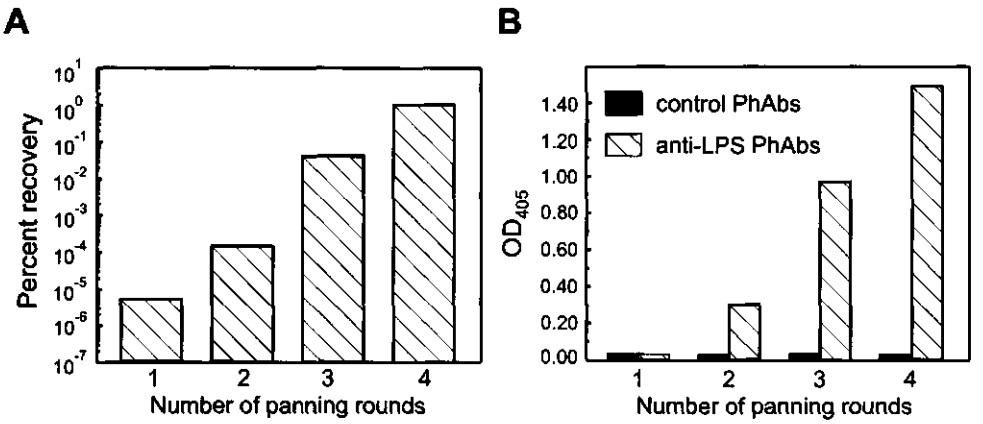

Figure 2.1. Recovery and specificity of LPS-binding phage-antibodies in 4 sequential rounds of panning. PhAbs were applied to LPS coated tubes and allowed to bind. Bound PhAbs were eluted after washing and used to infect *E. coli*. After phage rescue, with helper phage, the PhAbs (anti-LPS enriched) were used for a new round of panning. The number of applied and recovered PhAbs was counted and the recovery was plotted for each subsequent panning round (**A**). A phage-ELISA was done (**B**) with equal amounts of the selected anti-LPS PhAbs and compared to comparable amounts of control PhAbs (derived from a panning against another antigen) to show that the binding was LPS specific.

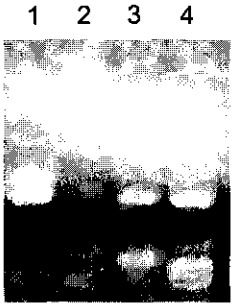

Figure 2.2. Characterization of four anti-LPS clones by *Mva*I-RFLP finger printing of the scFv encoding DNA: lane 1, anti-LPS 3; lane 2, anti-LPS 8; lane 3, anti-LPS 12 and anti-LPS 13, lane 4.

Characterization of monoclonal LPS-binding scFv

Four bacterial clones, which produced scFvs with high activity in the cMyc-ELISA (designated anti-LPS 3, anti-LPS 8, anti-LPS 12 and anti-LPS 13), were further characterized. The variability within the scFv genes was established using RFLP of PCR amplified DNA. Four different *Mva*I-restriction patterns were observed (Fig. 2.2). Although the patterns of anti-LPS 12 and anti-LPS 13 looked quite similar, nucleotide DNA sequencing of plasmid DNA revealed that these scFvs were different (data not shown). The four different scFvs were purified by immobilized metal affinity chromatography. Anti-LPS 3 could only be purified in low amounts, 50 µg/l maximum yieldscore, and SDS-PAGE indicated that the bulk of this was degraded, as it did not have the expected molecular mass of 27 kDa (data not shown). This was in contrast to the scFvs anti-LPS 8, 12 and 13 that yielded up to 1.5, 3 and 2.5 mg of intact scFv-antibody per liter, respectively.

Purified scFvs anti-LPS 8, 12 and 13 gave high signals when tested in cMyc-ELISA with purified LPS (Fig. 2.3) and with *R. solanacearum* cells (data not shown), whereas a weak reaction was found with scFv anti-LPS 3. Similar results were obtained with IF. The scFvs anti-LPS 8, 12 and 13 but not scFv 3 gave bright fluorescent staining of *R. solanacearum* at 12.5 µg/ml (Fig. 2.4A) and brilliant fluorescence at 125 µg/ml (data not shown). It was confirmed that the scFvs indeed reacted with LPS of *R. solanacearum* by Western blotting, whereas no reaction with LPS from *R. picketti* was found (Fig. 2.4B).

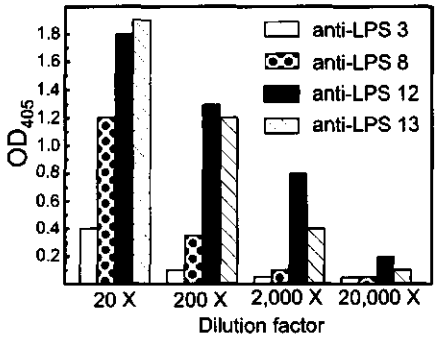

Figure 2.3. Reaction of purified monoclonal scFv antibodies (anti-LPS 3, anti-LPS 8, anti-LPS 12 and anti-LPS 13) with LPS in ELISA. ELISA plates were coated with LPS derived from *R. solanacearum*. ScFv antibodies were applied and the binding to LPS was detected by subsequent incubations with anti-cMyc MAbs, Rat anti-Mouse PAbs conjugated with alkaline phosphatase and para-nitrophenylphosphate.

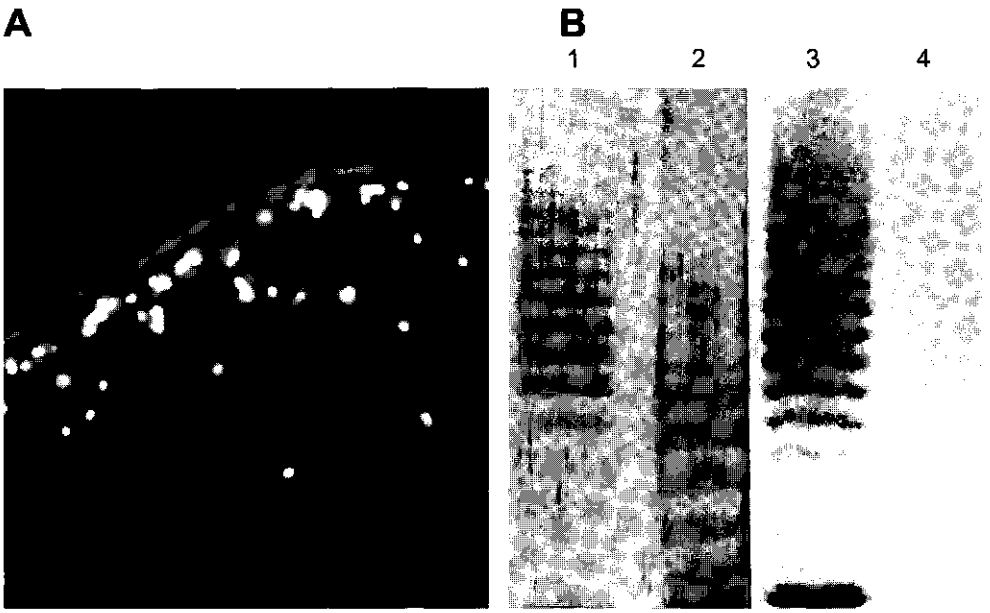

Figure 2.4. Immunofluorescence staining of whole *R. solanacearum* bacteria (**A**), showing that a surface epitope is recognized by scFv anti-LPS12 (12.5 µg/ml) and a Western blot (**B**), showing that the epitope which is recognized is located on the LPS of *R. solanacearum*. Purified LPS, derived from *R. solancearum* (tracks 1 and 3) and *R. picketti* (tracks 2 and 4) was blotted onto nitrocellulose and stained with silver (tracks 1 and 2) or with scFv anti-LPS 12 (tracks 3 and 4), respectively.

The detection limit for *R. solanacearum* of scFv anti-LPS 12, the most reactive of the isolated scFvs, was comparable to polyclonal antiserum PcA-9523. In a double antibody sandwich ELISA, both could detect as few as 4 x 10^4 cells ml^{-1} when in pure culture (Table 2.1). No difference in sensitivity was observed between the chromatogenic substrate p-NPP and the fluorogenic substrate FDP. The signals obtained were higher for PcA-9523 than for scFv anti-LPS 12, but the background value of scFv anti-LPS 12 was lower, especially when FDP substrate was used instead of p-NPP.

For evaluation of scFv anti-LPS 12 for routine application, *R. solanacearum* cells were also diluted in a potato tuber extract and compared to serial dilutions of a pure culture. The lowest concentration of *R. solanacearum* cells that could be detected in the sandwich ELISA with scFv anti-LPS 12 was 5 x 10^3 cells ml^{-1} in the potato tuber extract (Table 2.2). Even though high numbers of saprophytic bacteria were present in the tuber extract, only slightly reduced signals were obtained and the sensitivity was comparable to the pure culture.

Table 2.1. Comparison of scFv anti-LPS 12 and polyclonal antiserum PcA 9523 for their detection limits for *R. solanacearum* race 3 in ELISA. Bacteria, strain 1609, were suspended in PBS and applied in different concentrations. The reactions were visualized using the chromatogenic substrate p-NPP or the fluorogenic substrate fluoresceindiphosphate (FDP).

R. solanacearum concentration (cfu/ml)	ELISA substrates			
	p-NPP (OD_{405} value)		FDP (relative fluorescence)	
	scFv 12[a]	PcA-9523[b]	scFv 12[c]	PcA-9532[c]
$4x10^5$	2.8[d]	2.9	3.3	2.9
$4x10^4$	0.86	0.9	0.7	2.5
$4x10^3$	0.21	0.42	0.14	0.83
$4x10^2$	0.15	0.35	0.10	0.69
0	0.16	0.20	0.09	0.68

[a,b,c]Incubation times with substrate were 120, 40 or 80 minutes, respectively.

[d]The data were obtained from a representative experiment and the standard deviations (n=2) were smaller than 0.1 for all presented data. ELISA signals which exceeded at least three times the background value were regarded as a positive response.

Table 2.2. Values obtained in an ELISA, with scFv anti-LPS 12 against *R. solanacearum* race 3, using the chromatogenic substrate p-NPP or the fluorogenic substrate fluoresceindiphosphate (FDP). Different concentrations of *R. solanacearum* bacteria, strain 1609, were suspended in PBS (pure culture) or in a potato tuber extract and the detection limits were assayed with a double antibody sandwich ELISA.

R. solanacearum concentration (cfu/ml)	ELISA substrates (units)			
	p-NPP (OD_{405})[a]		FDP (relative fluorescence)[a]	
	pure culture	tuber extract	pure culture	tuber extract
$5x10^5$	> 3[b]	> 3	3.1	2
$5x10^4$	1.4	1.1	1.9	1.6
$5x10^3$	0.44	0.44	0.51	0.31
$5x10^2$	0.33	0.36	0.19	0.18
0	0.28	0.31	0.11	0.1

[a]Incubation time with substrate was 120 minutes.

[b]The presented data describe a typical experiment and the standard deviations (n=2) were smaller than0.1. ELISA signals which exceeded at least three times the background value were regarded as a positive response.

Testing of the scFvs against several *R. solanacearum* strains, both in ELISA and IF (Table 2.3), revealed that they reacted with all strains of race 3, but only with some of the race 1 strains. No difference was observed between the specificities of the anti-LPS 8, anti-LPS 12 and anti-LPS 13 scFv antibodies, and therefore only the results with scFv anti-LPS 12 are shown.

Table 2.3. Comparison of the reactivity of monoclonal scFv (anti-LPS12) and polyclonal antiserum (PcA 9523) with *R. solanacearum* in ELISA and IF.

Strain	Isolated in	IPO	Original	Race	Biovar	ELISA[a]		IF[b]	
	Country	number	number			ScFv	PcA	ScFv	PcA
R. solanacearum	Brazil	1657	PD1414	1	1	-	+	-	nd
R. solanacearum	Costa Rica	1653	PD507	1	3	+	+	+	+
R. solanacearum	Panama	1655	PD1255	1	3	-	+	-	nd
R. solanacearum	France	1656	PD1258	1	3	-	+	-	nd
R. solanacearum	France	1669	PD1256	1	3	-	+	-	nd
R. solanacearum	Indonesia	1670	PD278	1	3	-	+	+	nd
R. solanacearum	Hawaii	1654	PD508	1	4	-	+	-	nd
R. solanacearum	Japan	1658	PD1419	1	4	+	+	+	+
R. solanacearum	Panama	1665	PD1445	2	1	-	+	-	nd
R. solanacearum	Costa Rica	1666	PD1446	2	1	-	+	-	nd
R. solanacearum	Egypt	933	PD445	3	2	+	+	+	+
R. solanacearum	Netherlands	1608	PD2762	3	2	+	+	+	+
R. solanacearum	Netherlands	1609	PD2763	3	2	+	+	+	+
R. solanacearum	Netherlands	1610	PD2764	3	2	+	+	+	+
R. solanacearum	Egypt	1661	PD426	3	2	+	+	+	+
R. solanacearum	India	1662	PD1254	3	2	+	+	+	+
R. solanacearum	France	1663	PD1260	3	2	+	+	+	+
R. solanacearum	Chile	1664	PD1408	3	2	+	+	+	+
R. solanacearum	Netherlands	1676	PD2822	3	2	+	+	+	+
R. solanacearum	Netherlands	1677	PD2828	3	2	+	+	+	+
R. solanacearum	Netherlands	1678	PD2831	3	2	+	+	+	+
R. solanacearum	United Kingdom	1681	PD2474		2	+	+	+	+
R. solanacearum	United Kingdom	1684	PD2477		2	+	+	+	+
R. solanacearum?	Unknown	1652	NIZO96			-	+	nd	nd
R. solanacearum	France	1711	1310			+	+	+	+
R. solanacearum	France	1712	1361			+	+	+	+

[a]ELISA signals: - = reaction below background; and + = strong reaction (at least three times above background).

[b]IF signals: - =no fluorescence; + = strong fluorescence; and nd = not done.

Strains were obtained from Dr. J.D. Janse [Plant Protection Service (PD), Wageningen, the Netherlands] and Mrs A.C. Le Roux (INRA, Rennes, France).

The cross-reactivity of the recombinant antibodies was compared to PcA-9523, using a panel of plant pathogenic and saprophytic bacteria (Table 2.4) which had been shown to cross-react with polyclonal antisera in ELISA, IF or immunofluorescent colony staining. In ELISA and IF tests, scFvs cross-reacted with the banana blood disease bacterium (BBD), *R. syzygii*, *Serratia fonticola*, two strains belonging to the *Enterobacteriaceae* and with an unidentified bacterial strain: 1713, (Table 2.4). As was shown in Table 2.4, PcA-9523 cross-reacted with several bacterial strains that were not recognized by the scFvs. Thus, the specificity of the scFvs was higher than that of PcA-9523, the polyclonal antiserum that is used routinely for the detection of *R. solanacearum* in the Netherlands.

Table 2.4. Comparison of the reactivity of monoclonal scFv (anti-LPS12) and polyclonal antiserum (PcA 9523) with various bacterial strains in ELISA and IF.

Strain				Immunoassay			
Family[a], genus or species	Origin	IPO number	Original Number	ELISA[b] ScFv	ELISA[b] PcA	IF[c] ScFv	IF[c] PcA
Ralstonia solanacearum	Netherlands	1609	PD2763	+	+	+	+
Banana blood disease	Sulawesi	S303	PD2100	+	+	+	+
Ralstonia picketti	United Kingdom	1720	Pr1150	-	+	-	+
Ralstonia syzygii	Indonesia	S302	PD2093	+	+	+	+
Pseudomonas marginalis pv. *Marginales*	France	1714	CFBP1538	-	+	-	-
Unknown	France	1713	1531	+	+	-	-
Ochrobactrum antropi	Spain	1682	1521101	-	-	-	-
Rathayibacter tritici	Spain	1690	15803	-	+	-	+
Bacillus macerans	Spain	1691	15803	-	-	-	-
Aureobacterium liquefaciens	Spain	1692	15807	-	+	-	-
Aureobacterium liquefaciens	Spain	1693	158010	-	+	-	+
Serratia fonticola	Spain	1694	1534.c	+	+	+	+
Ralstonia	Netherlands	1723	PD2778	-	-	-	+
Unknown	France	1715	R3	-	-	-	+
Ochrobactrum antropi	Unknown	S306	PD2808	-	-	-	+
Enterobacteriaceae	Netherlands	S339	PK15a	+	+	+	+
Rhodococcus	Netherlands	S340	PK10a	-	-	-	-
Xanthomonas maltophilia	Netherlands	S341	PK06b/b	-	-	-	-
Erwinia herbicola	Netherlands	S343	PK18a	-	-	-	-
Enterobacteriaceae	Netherlands	S345	PK04a	-	-	-	+
Bacillus	Netherlands	S346	PK2/455	-	-	-	+
Rhodococcus	Netherlands	S347	PK2/76c	-	-	-	-

Family[a], genus or species	Origin	IPO number	Original Number	ELISA[b]		IF[c]	
				ScFv	PcA	ScFv	PcA
Unknown	Sweden	S348	PK2/76b	-	-	-	+
Bacillus polymyxa	France	1721	CFBP1954	-	-	-	-
Ochrobactrum antropi	United Kingdom	1722	T-3b-7	-	+	-	-
Sphingobacterium multivorum	Unknown	1717	R1c-3a	-	-	-	-
Erwinia carotovora subsp. *Carotovora*	France	1710	233	-	-	-	+
Burkholderia andropogonis	United Kingdom	1704	NCPPB1127	-	+	-	-
Burkholderia caryophylli	United Kingdom	1705	NCPPB2151	-	+	-	+
Burkholderia caryophylli	United Kingdom	1701	NCPPB353	-	-	-	+
Burkholderia cepacia	United Kingdom	1702	NCPPB945	-	-	-	-
Burkholderia cepacia	United Kingdom	1703	NCPPB946	-	+	-	-
Burkholderia cepacia	Unknown	1718	R5d-1	-	-	-	-
Burkholderia glumae	United Kingdom	1708	NCPPB3708	-	+	-	+
Burkholderia plantarii	United Kingdom	1707	NCPPB3590	-	-	-	+

[a]Fatty acid analysis was used for strain identification and the best matches with the database of the Microbial Identification system are given. Sometimes, characterization was only up to family level as no closer match (at least 50 % identity) was found. Strains were obtained from Dr. J.D. Janse [Plant Protection Service (PD), Wageningen, the Netherlands], Dr. M.M. Lopez, IVIA, Valencia, Spain), Mrs A.C. Le Roux (INRA, Rennes, France) and Dr. J.G. Elphinstone (CSL, York, UK.).

[b]ELISA signals: - = reaction below background; and + = strong reaction (at least three times above background).

[c]IF signals: - =no fluorescence; + = strong fluorescence; and nd = not done.

DISCUSSION

Four different scFv antibodies were isolated from a combinatorial antibody library and evaluated for their utilization in a diagnostic assay for the detection of *R. solanacearum* race 3. Three of these, anti-LPS 8, anti-LPS 12 and anti LPS 13 were promising candidates for routine application, as was based on their ease of production in *E. coli* and their specificity in IF and ELISA. The affinity of anti-LPS12 was sufficient for sensitive detection of *R. solanacearum*, as threshold levels between 5 x 10^3 and 4 x 10^4 bacterial ml^{-1} were reached in ELISA and bright fluorescent staining was achieved in IF.

The selected antibodies showed a higher degree of specificity than PcA-9523, the polyclonal antiserum that is used presently in routine testing. Apparently, the recognized LPS epitopes do not occur as frequently in the bacteria tested (Table 2.4) as other epitopes recognized by PcA-9523. Interesting is that all four different scFvs recognize the same bacterial strains and probably react with the same epitope. However, this was not validated, as no epitope mapping was carried out. The cross-reactions (Table 2.4) observed with BBD and *R. syzygii* will not compromise a diagnostic test for potato brown rot because these species are

not present in temperate areas where this test will be used. In contrast, the cross-reactions observed with the four other saprophytic bacteria from potato and soil can possibly cause false-positive results because they have been isolated in temperate areas. However, as the number of cross-reacting species is reduced relative to the PAbs in PcA-9523, it is to be anticipated that the use of these antibodies in seed testing will result in a decreased number of false positives.

The sensitivity observed in ELISA, comparable to high quality PAbs, is promising. In IF relatively high quantities of scFv-antibody were required to obtain the same signal as the PAbs. This is probably because the scFv-antibodies have one antigen-binding site and bind monovalently to the antigen. In contrast, full size antibodies have two antigen binding sites that can bind with two epitopes simultaneously. According to Neri *et al* [19], this avidity effect can increase binding up to 100-fold and results in an improved performance in immunoassays. Single-chain antibodies can be engineered in a format allowing bivalent binding [21]. Bivalent molecules can also be established through a genetic fusion with *E. coli* alkaline phosphatase, a dimeric enzyme [13, 14]. Thus, if one of these avidity effects is added, it may further increase the sensitivity of the immunoassay. Alkaline phosphatase may be the most convenient choice for detection purposes. The production of scFv-enzyme fusion proteins may result in an improved reproducibility for the detection of antigens in various immunoassays since the variation due to repeated antibody enzyme conjugation is avoided.

Engineering the format of scFvs might also be advantageous for the development of routine assays for the detection of *R. solanacearum*. Single-chain antibodies are small molecules that are often inactivated upon coating to ELISA plates [12]. Fusion to large proteins like gluthatione-S-transferase, amphipathic helices or to other immunoglobulin domains may enhance the coating efficiency while retaining the full binding activity [12].

The monoclonal recombinant scFvs described here are applicable in routine assays for *R. solanacearum* race 3, even though some cross-reactions with other species were observed. The selection of other anti-LPS scFvs that do not cross-react with these strains remains an option. The difficulty lies in the nature of the LPS of the cross-reacting bacteria. Although lipopolysaccharides are highly variable molecules, the possibility can not be excluded that LPS epitopes of cross-reacting bacteria are at least partially identical to the target bacteria. For the isolation of even more specific antibodies consideration may be given to outer membrane proteins or extracellular polysaccharides as antigens. We used a straightforward selection protocol that resulted in the most specific antibodies found thus far for *R. solanacearum* (biovar 2, race 3). However, phage display allows easy adaptation of the selection protocols, such as the inclusion of cycles of negative (against unwanted bacteria) and positive selection (for different races). In addition, the present antibody libraries cover the complete immune repertoire, in contrast to the hybridoma technology where only a part of the immune repertoire is tested. Antibody phage display in combination with protein engineering is highly promising for the selection of tailor-made monoclonal antibodies and can be combined with the design of improved immunoassays for phytopathogenic bacteria.

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Chapter 3

Selection of beet necrotic yellow vein virus specific single-chain Fv antibodies from a semi-synthetic combinatorial antibody Library

ABSTRACT

Methods for the generation of monoclonal antibodies against plant viruses are limited because current hybridoma techniques do not allow efficient exploitation of the immune repertoire. Moreover, the immunization procedures often lead to a bias towards an immunodominant contaminant in the immunogen preparation and not to the plant virus itself. The selection of six different single-chain antibody variable fragments (scFv) against beet necrotic yellow vein virus from a semi-synthetic human combinatorial antibody library showed the feasibility of the phage display system. No bias towards minor contaminants in the purified virus preparation was observed in ELISA, as all the selected scFvs reacted only with beet necrotic yellow vein virus infected plant homogenates. In addition, two of the isolated beet necrotic yellow vein virus-specific scFvs could be produced in *E. coli* as a scFv fusion protein with alkaline phosphatase, and were applied in ELISA as specific ready to use antibody-enzyme conjugates. Because of their specificity, these antibodies have potential to be used in sensitive diagnostic assays for routine testing for beet necrotic yellow vein virus in sugar beets.

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INTRODUCTION

Most diagnostic assays for plant viruses utilize polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs). MAbs do have some advantages over PAbs. They are homogeneous and well defined reagents that allow standardization of assays between different laboratories [28,29]. Due to the fact that MAbs recognize single epitopes they may give less cross-reactions, discriminate between viral strains [26,27] and MAbs directed against conserved epitopes may allow grouping of virus isolates for epidemiological and taxonomical studies.

It is difficult to raise MAbs to some plant viruses because minor contaminants in the isolated virus fraction exhibit immunodominance [5]. This was shown to be the case for beet necrotic yellow vein virus (BNYVV), a furovirus causing rhizomania [24]. During purification the virus aggregates with host cell constituents [21] and only a few MAbs were obtained reacting specifically with BNYVV, while the majority of the MAbs reacted with healthy plant antigens. It is noteworthy that polyclonal antisera collected at the time of spleen removal had high virus-specific and low anti-healthy plant sap titers. The same phenomenon has been observed in developing MAbs to cauliflower mosaic virus [5] and blueberry red ringspot virus [6].

Various methods have been described to reduce the immune response to plant contaminants to enhance the yield of virus-specific MAbs. For instance, the induction of immunological tolerance in neonatal mice [8], the use of immunosuppressive agents like cyclophosphamide [14], or use of antigen based B-cell selection systems [20]. The common drawback of these methods is that the conditions have to be established for each virus.

Current hybridoma methods [12] for the generation of MAbs against plant pathogens do not satisfactory exploit the immune repertoire. While the antibody repertoire is estimated to consist of over 10^8 different antibodies, only a few thousand different hybridoma clones are obtained, of which on average less then 1% produce antigen binding antibodies. Thus, the chance that a useful hybridoma derived MAb will be obtained is low. This diversity and efficiency problem was recently addressed by advances in molecular immunology. Forced cloning of DNA, encoding antibody variable heavy (V_H) and light (V_L) chain domains with reverse transcription PCR [19,23], allowed amplification of the antibody repertoire encoding sequences. Expression of V_H and V_L domains as a single-chain Fv (scFv) fusion protein (Fig. 1.3) in *E. coli* was made possible by joining them with a flexible linker peptide [10]. Cloning of a pool of scFv encoding genes, in which V_H and V_L were randomly combined, enabled the generation of large combinatorial antibody libraries which have a diversity comparable to the natural immune repertoire [9,13]. This, in combination with the display of functional antigen binding fragments on the tips of filamentous phage, created a powerful system [3,7,15] to obtain specific MAbs. The phage display system (Fig. 1.6) allows direct selection of rare specificities from combinatorial antibody libraries through successive rounds of phage growth and selection for antigen binding. For instance, Bradbury and co-workers [1] showed the

feasibility of this technique by selecting antigen binding phages from a pool of non binding phages, even at a ratio of one binding in 10^9 irrelevant phages. In addition, the phage display system may also prove to be a more universal method to obtain specific MAbs, since the antibody specificities are not biased towards immunodominant epitopes.

To study the versatility of the phage display system, we have applied it to BNYVV. This virus was chosen as a model system because it is a classic example of the difficulties outlined in raising specific MAbs. We examined the feasibility of the phage display system in isolating a diverse repertoire of BNYVV-specific antibody fragments from a semi-synthetic combinatorial antibody library [18].

MATERIALS AND METHODS

Bacterial strains

E. coli strains used for the isolation of recombinant antibodies, were TG1 (K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*D5/F'*tra*D36, *pro*A$^+$B$^+$, *lacI*^{q}, *lacZ*ΔM15) for selection of specific phage-antibodies and HB2151 (K12, *ara*, Δ*lac-pro*), *thi*/F'/*pro*A$^+$B$^+$, *lacI*^{q}ZΔM15) for expression of soluble scFv-antibody fragments. HB2151 bacteria were grown at 30°C in 2TY containing 100 μg ml^{-1} ampicillin and 2% (w/v) glucose [22]. When the OD_{600} reached 0.5, the bacteria were pelleted and the supernatant was discarded. The bacteria were resuspended in 2TY + AMP (100 μg/ml), induced with 1 mM IPTG and grown for 18 h at 16°C.

Purification of beet necrotic yellow vein virions

Beet necrotic yellow vein virus (Dutch isolate) was purified from mechanically inoculated *Chenopodium quinoa* according to a slightly modified protocol from Putz and Kuszala [21]. Infected *C. quinoa* leaves (200 g) were homogenized in a mixture of 400 ml CCl_4 and 800 ml extraction buffer (0.1 M Tris/HCl buffer pH 9 containing 0.14 M NaCl; 5% (v/v) ethanol) and 2% (w/v) polyvinylpyrrolidon. The mixture was stirred for 15 min at room temperature (RT) and then centrifuged for 15 min at 5,000 x g (GSA rotor, Sorvall). To the collected supernatant, 5% (w/v) PEG-8000 was added and stirred at RT for 15 min and subsequently at 4°C for 90 min. The virions were pelleted at 16,000 x g (GSA, Sorvall) for 20 min. After resuspending the pellets in 100 ml extraction buffer and stirring overnight at 4°C, sucrose was added to 3.3% (w/v) and the mixture was centrifuged at 27,500 x g for 20 min (SW28, Beckman) to remove large aggregates. Subsequently the virions were pelleted at 110,000 x g (SW28, Beckman) for 2 h through a 3 cm layer of 20% (w/v) sucrose in extraction buffer. The pellets were resuspended in 50 ml extraction buffer and, after stirring for 30 min at RT, centrifuged for 10 min at 1,600 x g to remove insoluble precipitates. Half of the supernatant was adjusted to a density of 1.1 g/cm^3, half to 1.3 g/cm^3 with $CsCl_2$. The two halves were layered 1:1 and centrifuged at 154,000 x g (SW41Ti, Beckman) for 18 h. The virions containing band was collected and dialyzed overnight against 0.1 M Tris/HCl buffer pH 9

containing 0.14 M NaCl and then centrifuged for 5 min at 12,000 x g to remove aggregates. The virion concentration was determined with a Beckman photospectrometer using a formula [(1552 x A_{280}) + (-757.3 x A_{260})], described by Warburg and Christian [30], which gives the protein concentration in μg ml^{-1}. The concentration was adjusted to 1 mg ml^{-1} with 0.1 M Tris/HCl buffer pH 9 containing 0.14 M NaCl and 1 ml aliquots were frozen in liquid nitrogen and stored at -74°C until use.

Expansion of the antibody library

From a stock of the combinatorial library, which was constructed by Nissim et al [18], 50 μl was plated on a 240 x 240 mm minimal plate [22] to which 1mM thiamin was added. The bacteria were grown overnight at 30°C, scraped from the plate and resuspended in 50 ml of 2TY containing 100 μg ml^{-1} ampicillin and 1% (w/v) glucose. A freezer stock [22] was prepared from 10 ml of these bacteria. One bacterial OD_{600} unit was taken from the remaining 40 ml, the volume adjusted to 10 ml by adding of 2TY containing 100 μg ml^{-1} ampicillin and 1% (w/v) glucose, and the bacteria were grown at 37°C while shaking (250 rpm). When an OD_{600} of 0.5 was reached, 10^{11} helper phages (M13K07, Pharmacia, Uppsala, Sweden) were added. The 15 ml tube containing the mixture was put in a waterbath without shaking to allow for optimal infection. After 30 min the bacteria were pelleted (2,100 x g, 10 min) and resuspended in 25 ml 2TY containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin. The bacteria were transferred to a 1 liter Erlenmeyer, with 225 ml of prewarmed (37°C) 2TY containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin, and were grown for 18 h at 30°C while shaking (250 rpm).

Preparation of phage-antibodies for panning (large-scale production)

The 250 ml overnight culture was harvested and the bacteria were removed by centrifugation (10,000 x g, 20 min, GSA, Sorvall). The phages in the supernatant were precipitated by adding 50 ml of 20 % (w/v) PEG-6000/ 2.5 M NaCl and mixed thoroughly for 1 h at 4°C. The precipitated phages were pelleted (8,000 x g, 40 min) and resuspended in 20 ml of sterile H_2O. Precipitation was repeated by adding 4 ml of PEG/NaCl and mixed for 20 min at 4°C. The phages were pelleted (17,000 x g, 10 min; SS34, Beckman), resuspended in 2.5 ml sterile H_2O and the stock was stored at 4°C for further use. Usually 5 x 10^{13} phages were produced, as was established by plating TG1 bacteria on selective ampicillin plates after infection with serial dilutions of the phage suspension.

Panning procedure

Immunosorbent tubes (Maxi-sorb, Nunc) were coated with 40 μg BNYVV (10 μg ml^{-1} in 50 mM $NaHCO_3$, pH 9.5) for 2 h on a rollerbench at 25°C and for another 18 h at 4°C. The tubes were washed 3 times with PBS and blocked with PBM (PBS containing 2% (w/v) skimmed milk powder) for 30 min. Simultaneously, 2 ml of phage-antibodies was mixed with 2 ml of 2

x PBM and preincubated for 30 min. After removing the blocking solution from the tubes, 4 ml of the phage-antibodies containing PBM was added to an antigen-coated tube. Phage-antibodies were allowed to bind to BNYVV for 30 min on a roller bench and for another 90 minutes without rotation. Free phages were removed by washing the tubes 20 times with 4 ml PBS containing 0.1% (v/v) Tween-20 and for another 20 times with 4 ml PBS to remove the detergent. After washing the tubes were rinsed with 4 ml 0.1 M Tris/HCl pH 8.9 containing 0.14 M NaCl. Bound phages were eluted by adding 1 ml of a 0.5 mg ml^{-1} BNYVV solution in 0.1 M Tris/HCl pH 8.9 containing 0.14 M NaCl to the tube and incubated for 45 min with rotation, followed by a wash with 0.75 ml PBS. The BNYVV-eluted phages and phages in the PBS washing were pooled. One ml aliquot, of the BNYVV-eluted phages, was used to infect 9 ml of *E. coli* TG1 bacteria, for 30 min at 37°C in a water bath. The infected *E. coli* cells were pelleted (2,100 x g, 10 min) and subsequently resuspended in 1 ml of 2TY, containing 100 µg ml^{-1} ampicillin and 1% (w/v) glucose. From these suspensions 50 µl aliquots were taken and plated in serial dilutions on 2TY agar plates containing 100 µg ml^{-1} ampicillin and 1% (w/v) glucose to establish the number of eluted phages and to make freezer stocks [22] of TG1 bacteria which were growing in single colonies. The remaining 950 µl was plated separately on 240 x 240 mm minimal plates [22] to which 1 mM thiamin was added, and grown for 18 h at 30°C. Further enrichment of BNYVV-specific phage-antibodies was achieved by three additional panning rounds, which were performed according to figure 1.6.

Preparation of phage-antibodies for ELISA (medium-scale production)

From a streak of the bacterial freezer stocks, prepared during the panning procedure, single colonies were tooth picked and plated on minimal plates [22] to which 1mM thiamin was added. The bacteria were grown for 18 h at 30°C, scraped from the plate and resuspended in 2 ml of 2TY containing 100 µg ml^{-1} ampicillin and 1% (w/v) glucose. One bacterial OD_{600} unit was adjusted to 1 ml by adding of 2TY containing 100 µg ml^{-1} ampicillin and 1% (w/v) glucose, and 2 x 10^{10} helper phages (M13K07, Pharmacia). The 1.5 ml Eppendorf tube containing the mixture was put in a waterbath for 30 min without shaking to allow for optimal infection at 37°C. The bacteria were pelleted (8,000 x g, 10 min) and resuspended in 750 µl 2TY containing 100 µg ml^{-1} ampicillin and 50 µg ml^{-1} kanamycin. The bacteria were transferred to a 24-well culture plate and grown for 18 h at 30°C while shaking (100 rpm). The 750 µl overnight culture was harvested and the bacteria were removed by centrifugation (8,000 x g, 10 min). If necessary, the phages in the supernatant were precipitated by adding 150 µl of 20% (w/v) PEG-6000/2.5 M NaCl and mixing well for 1 h at 4°C. The precipitated phage-antibodies were pelleted (8,000 x g, 10 min), resuspended in 250 µl of PBM and stored at 4°C until further use.

Preparation of phage-antibodies for ELISA (small-scale production)

After each panning round, 100 single colonies were picked and inoculated in 100 µl of 2TY containing 100 µg ml^{-1} ampicillin and 1% (w/v) glucose in a well of a 96-well culture plate. To study the effect of the panning also 100 single colonies were picked from the original library before any selection was carried out (Panning round 0). The bacteria were grown shaking (200 rpm) for 18 h at 30°C. From these overnight cultures 5 µl was taken, inoculated in a new 96-well plate well, containing 100 µl of 2TY with 100 µg ml^{-1} ampicillin and 1% (w/v) glucose and grown shaking for 1 h at 37°C. To allow super-infection, 50 µl aliquots of 2TY containing 100 µg ml^{-1} ampicillin, 1% (w/v) glucose and 2.5 X 10^9 helper phages (M13K07, Pharmacia) were added to each well and incubated for 30 min without shaking and for 1 h with shaking (200 rpm) at 37°C. The bacteria were pelleted (2,800 x g, 10 min) and resuspended in 200 µl 2TY containing 100 µg ml^{-1} ampicillin and 50 µg ml^{-1} kanamycin. The bacteria were grown shaking (200 rpm) at 30°C for 18 h, removed by centrifugation (2,800 x g, 10 min) and the phage-antibody containing supernatant was stored at 4°C until further use.

Phage-ELISA

The phage-ELISA was essentially carried out according to Clark and Adams [4] and to Tijsen [25]. Briefly, ELISA plates (Labstar, Costar, Cambridge, UK) were coated overnight with 100 µl of polyclonal rabbit-anti-BNYVV antibodies (3 µg IgG ml^{-1} in 50 mM $NaHCO_3$, pH 9.5) at 4°C. The plates were washed with PBST (PBS containing 0.1% (v/v) Tween-20) and blocked with PBM for 30 min at 37°C at 200 µl per well. The microtiter plates were washed twice with PBST and incubated for 1 h at RT with 100 µl/well of a *C. quinoa* leaf homogenate (50 mg dry leaf material ml^{-1} in PBM), with or without BNYVV lesions. After each incubation step the microtiter plates were washed four times with PBST. The plates were subsequently incubated for 1 h at RT with phage samples, diluted 1:1 in PBM; with polyclonal mouse-anti-M13 antibodies, diluted 1:10,000 in PBM; and finally with polyclonal rat-anti-mouse antibodies conjugated to alkaline phosphatase (Jackson Immuno-Research Laboratories, Inc., Westgrove, PA) diluted 1:2500 in PBM at 100 µl per well. The ELISA was developed by adding 100 µl p-nitrophenylphosphate substrate per well and absorbance readings were made at 405 nm, usually within 60 min. A reaction in phage-ELISA was considered to be positive if the threshold value (mean of the background + three times the standard deviation) was exceeded.

***Mva*I-fingerprinting**

Fingerprinting was performed on PCR amplified scFv-DNA using the restriction enzyme *Mva*I. Single colonies were tooth picked and grown for 4 h in 2TY containing 100 µg/µl ampicillin and 1% (w/v) glucose. The bacteria were boiled for 3 min and pelleted at 14.000 x g for 10 min. From the supernatant 2 µl was added to a 48 µl PCR mix containing 2.5 µM dNTPs; 0.25 U Super Taq DNA polymerase (HT Biotechnology, Cambridge, UK); 10 µM

Forward primer (5'-AGG AAA CAG CTA TGA CCA TGA TTA CGC CAA G-3') and 10 µM Backward primer (5'-GCC CAA TAG GAA CCC ATG TAC CGT AAC ACT G-3'); 2 mM $MgCl_2$ and 50 mM Tris/HCl, pH 8 and 25 cycles (1 min 94°C; 2 min 72°C) were performed on a thermal cycler (Perkin Elmer). From the PCR mix, 20 µl was added to 36.5 µl H_2O and 6,5 µl of buffer H (Boehringer, Mannheim, Germany). After mixing 2 µl (1U/µl) *Mva*I (Boehringer) was added and incubated 18 h at 37 °C. The *Mva*I digestion patterns were analyzed on a 3% FMC Metaphor agarose gel (Epicentre Technologies, Madison, USA).

Cloning of scFvs into pDAP2/S

The genes encoding the scFvs, BNY-8 and BNY-10, were PCR amplified using the forward and backward primers, *Hin*dIII and *Not*I digested, gel purified, ligated into *Hin*dIII/*Not*I digested pDAP2/S vector [11] and transfected to *E. coli* TG1 bacteria. Transformed bacteria were picked and tested for the expression of scFv-alkaline phosphatase fusion-proteins through incubations with p-nitrophenyl phosphate.

Production of scFv-AP/S fusion-proteins

Cultures were grown and induced as described by Kerschbaumer and co-workers [11]. Pelleted bacteria (5,000 rpm in a GSA rotor during 20 min at 4°C) were resuspended in 1/20 volume (referring to the original culture size) of a 50 mM Tris/HCl pH 8 buffer (containing 30% sucrose and 1 mM EDTA). After incubation for 5 min at 0°C the bacteria were pelleted (10,000 rpm in a GSA rotor during 20 min at 4°C) and the produced proteins were extracted by an osmotic shock from the periplasm with 1/20 volume (referring to the original culture size) of 5 mM $MgSO_4$ and incubation for 45 min at 0°C. The bacterial debris was removed by centrifugation.

ELISA

An antigen coated plate (ACP)-ELISA was used to assess the specificity of the recombinant scFv-AP2/S fusion proteins in the osmotic shock fractions according to standard methods, in which the plates were washed four times between each incubation. Briefly, the wells of a 96 well microtiter plate were coated with the appropriate antigen in 0.1 M $NaCO_3$ pH 9.6) for 2 h at 37°C. After blocking with PBMT-5% (PBS containing 5% skimmed milk powder and 0.1% Tween-20) at 200 µl/well for 30 min at 37°C, the wells were incubated with scFv-AP2/S diluted in 100 µl of PBMT-1% for 1 h at 37°C. The reaction was visualized by a subsequent incubation with p-nitrophenylphosphate.

RESULTS

Selection of BNYVV binding clones from a phage-antibody library

To select BNYVV-specific monoclonal antibodies, scFv expressing phages were rescued from a semi-synthetic human antibody library containing 10^8 different antibody encoding phagemids [18], and subjected to panning. The rescued phages, containing 10^{13} phage-antibodies (PhAbs), were allowed to bind to immobilized BNYVV particles. Bound PhAbs, were eluted by specific elution with competing antigen. To monitor the efficiency of selection during the panning experiments, the phage recovery was measured after each panning round. Determination of the phage recovery (Fig. 3.1A) showed a clear enhancement. Especially in the third round where a strong increase in phage recovery, about 1,000 fold, was found. Because the fourth panning round did not show a further increase in phage recovery, a fifth selection round was not applied.

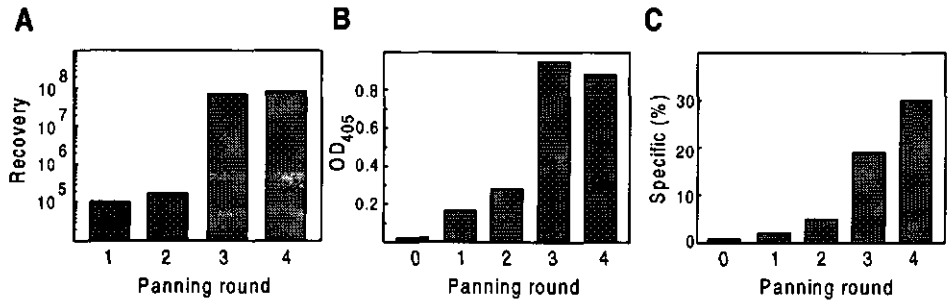

Figure 3.1. Recovery and specificity of BNYVV-binding phage-antibodies in four sequential rounds of panning. **A)** The number of recovered PhAbs was counted and the recovery was plotted for each subsequent panning round. **B)** Phage-antibodies were produced (medium-scale production) and comparable amounts were tested in polyclonal phage-ELISA. Phage-antibodies that were rescued from a stock of the antibody library before any selection had been applied served as negative control (panning round 0). **C)** In addition the percentage of BNYVV-specific phage-antibody producing clones (small-scale production) was shown by a monoclonal phage-ELISA. Colonies were counted positive in case the derived signal exceeded the mean (n=3) of the negative control pHen (non-scFv expressing phage) plus three times the standard deviation.

To verify if any of the selected phage-antibodies were BNYVV-specific, a polyclonal sandwich phage-ELISA was carried out with samples taken from the stocks of phage-antibodies which were prepared by large-scale production for each panning round. In this BNYVV-specific phage-ELISA, the polyclonal PhAbs showed increasing signals for subsequent rounds of panning (data not shown). The same stocks of polyclonal PhAbs were also tested for their reactivity using a phage-ELISA format in which purified BNYVV was coated directly to the plate. Although the signals from the sandwich ELISA were stronger than those obtained from the ACP phage-ELISA (data not shown), both ELISAs showed a similar increase. To ensure that differences in ELISA signals could be contributed to enhancements in specificity and not to degradation of the oldest samples during storage, and to allow the PhAbs obtained from the fourth selection round to be included in the ELISA as well, PhAbs were

rescued simultaneously from the freezer stocks of the bacteria. When comparable amounts of PhAbs were tested in ELISA, again an increase in BNYVV binding was observed up to the third panning round (Fig. 3.1B). This seemed to be a superlative level, as the signal showed no further increase after the fourth round of selection.

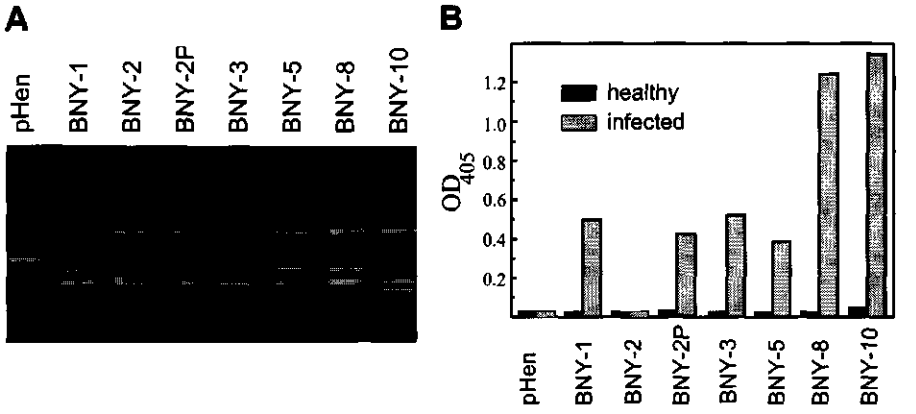

Figure 3.2. A) Restriction patterns of BNYVV binding clones which were obtained during selection. PCR-products (1100 Bp) of the DNA encoding the BNYVV-specific antibody fragments were digested with the restriction enzyme *Mva*I. The digested fragments were analyzed on a 3% FMC metaphor agarose gel. **B)** The specificity of the isolated PhAbs for BNYVV is shown in a phage-ELISA with homogenates of healthy and BNYVV-infected *C. quinoa* leaf material. Supernatants of infected *E. coli* cultures were tested directly for BNYVV specificity in the phage-ELISA after 1:5 dilution in PBM. Rescued (non-scFv expressing) phages, obtained from TG1 bacteria which were transformed with a no scFv containing phagemid (pHen), served as a negative control.

Characterization of BNYVV-specific phage-antibodies

The polyclonal character of the phage-ELISA does not give a clear image of the antibody specificity because of different growth rates and expression levels between individual clones. Therefore, 100 single clones were isolated from each panning round. The monoclonal phage-antibodies, produced by these individual clones, were tested separately in a phage-ELISA (Fig. 3.1C) and showed a strong increase in the number of positive clones from zero % before selection to as much as 30% after the fourth round of selection.

To investigate the diversity within the selected monoclonal phage-antibodies, a DNA fingerprint was carried out. The scFv encoding DNAs, of the clones that were positive in the phage-ELISA, were amplified by PCR and digested with the restriction enzyme *Mva*I. When the digestion products were analyzed on an agarose gel (Fig. 3.2A), a high variation was observed within the isolated monoclonal phage-antibodies. A total of six different *Mva*I-patterns was found within the positive clones. These monoclonal PhAbs were designated BNY-1, BNY-2, BNY-3, BNY-5, BNY8 and BNY-10, of which PhAb BNY-10 had become the most abundant PhAb after three rounds of selection. Up to 90% of the positive colonies possessed the same *Mva*I restriction pattern as PhAb BNY-10. In contrast, after four rounds of selection PhAbs like BNY-2, BNY-3 and BNY-5 had completely disappeared from the antibody pool.

To study the specificity of the selected MAbs towards healthy and BNYVV infected *C. quinoa* plants, one representative colony of each pattern was selected, streaked and after phage-antibody production tested for its reactivity in a phage-ELISA (Fig. 3.2B). Of the isolated PhAbs, five gave high reactions with BNYVV infected plants but no reaction with healthy *C. quinoa* plants in the BNYVV-specific phage-ELISA. The only MAb that failed to give a positive reaction in this ELISA corresponds to the restriction pattern of MAb BNY-2 that was found in a positive colony obtained from the first panning round but nowhere else. However, when the phage-ELISA was repeated with a PEG-purified and concentrated BNY-2 phage-antibody, a positive signal was found for BNYVV infected but not with healthy *C. quinoa* (Fig. 3.3B: BNY-2p).

Although the selected monoclonal phage-antibodies reacted specifically with BNYVV in a phage-ELISA, they failed when they were expressed in *E. coli* strain HB2151 as soluble scFv antibodies. When the HB2151 bacteria were fractionated after induction with IPTG, and tested by Western blotting for the presence of expressed scFvs, high signals were found within the cytoplasm (data not shown) but not within the periplasm. Apparently the transport of the scFvs to the periplasm is troublesome and the result is formation of inclusion bodies. An improvement was observed when the scFv genes of BNY-8 and BNY-10 were recloned into the expression vector pDAP2/S [11] which allowed the scFvs to be expressed as scFv-alkaline phosphatase fusion proteins (scFv-AP/S) that were subsequently secreted into the periplasm (Fig. 3.3). After extraction of the scFv-AP/S fusion proteins from the periplasm, specific signals were obtained in ELISA with extracts of BNYVV-infected plants (Fig. 3.4).

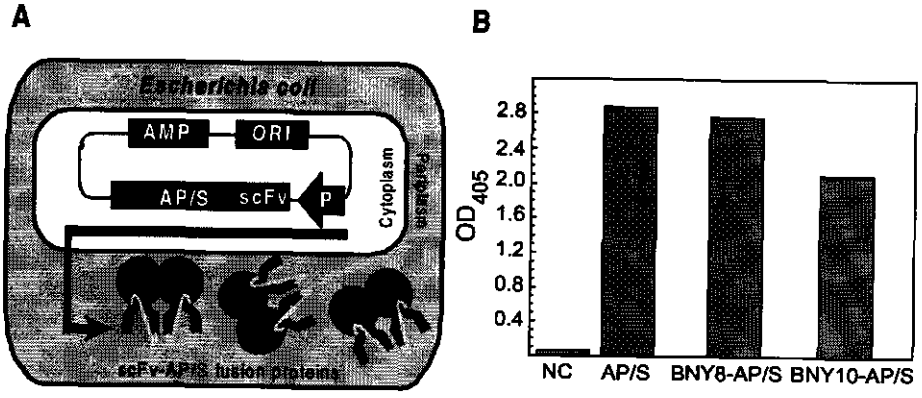

Figure 3.3. A) Expression of scFv-AP/S fusion proteins in *Escherichia coli*. **B)** Alkaline phosphatase activity in the osmotic shock fractions of non-fused AP/S, BNY8-AP/S and BNY10-AP/S was measured 10 minutes after the addition of 10 µl osmotic shock fraction to 100 µl of p-NPP substrate, where 10 µl of 5mM MgSO4 served as the negative control (NC).

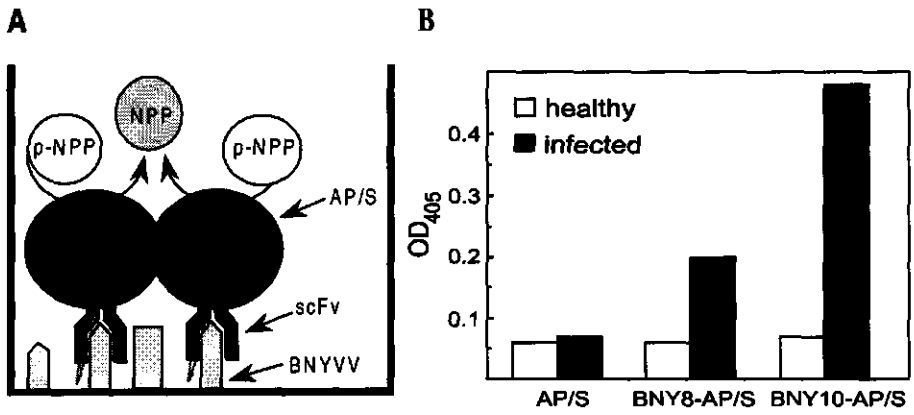

Figure 3.4. A) Outline of BNYVV-specific ELISA. The ELISA plate was coated either with a healthy or BNYVV infected *C. quinoa* homogenate. **B)** For detection of BNYVV, scFv alkaline phosphatase fusion proteins (BNY8-AP/S, BNY10-AP/S) or non-fused alkaline phosphatase (AP/S) were used and binding of the bifunctional antibody-enzyme conjugate was visualized with p-NPP substrate.

DISCUSSION

Six different BNYVV-specific monoclonal phage-antibodies were selected from a semi-synthetic human combinatorial antibody library [18], as was assayed by *Mva*I digestion of PCR amplified scFv encoding DNA. Each of these PhAbs reacts specifically with BNYVV from *C. quinoa* leaf homogenates in a sandwich ELISA. When the phage display technique is compared to the hybridoma technique a number of advantages are observed. First, laboratory animals are no longer required. Second, selection of antibodies from semi-synthetic combinatorial antibody libraries with phage display is much faster and less labor-intensive. Third, there is no bias towards certain minor plant contaminants in the antigen preparation. Finally, the specificity and affinity of the MAbs can be guided in a certain direction through manipulations during selection. For instance, high affinity antibodies can be obtained using low amounts of antigen or the specificity can be enhanced by elution with competing antigen.

Although elution with competing antigen gave a steep rise in BNYVV-binding PhAbs in the described selection procedure (Fig. 3.1C) and finally yielded a high PhAb diversity, it generally has the disadvantage to select for antibodies with high dissociation rates. Another disadvantage is the high amount of antigen that is required for elution: over 2 mg of purified BNYVV was required for four rounds of panning and elution. If the antigen is merely used for coating of the immunosorbent tubes then only 160 µg is needed. However, in cases where non-specific binding of the filamentous phage to the antigen is high, as was found for BNYVV in a pilot study, specific elution might be the only way to enrich for target-specific PhAbs during panning.

When the goal of selection is to obtain a diverse repertoire of antigen-specific antibodies with regard to recognition of different epitopes on the antigen or sets of scFvs recognizing the same epitope, care should be taken not to lose antibody diversity through applying too many rounds of selection. The percentage of positive clones improved with each subsequent round of panning (Fig. 3.2C) and 30% was reached after four rounds of selection. When more subsequent rounds of selection would have been performed the 100% would probably have been reached. However, a decrease in the diversity was observed after the third round of selection. This indicates that too many rounds of selection results either in antibodies with the highest affinity for the most abundant epitope or in antibodies which are produced at a faster rate and which are not necessarily the most specific.

The inability of *E. coli* to export the selected BNYVV-specific scFvs to the periplasm was observed. Several mutations are known which can enhance the transport of scFvs to the periplasm [17] and it might be worthwhile to introduce these mutations into the BNYVV-specific antibodies. The presence of an *E. coli* protein domain (AP/S) on the C-terminus of the scFv molecule already seemed to increase the stability and the folding efficiency of the soluble scFv. In addition, the dimeric character of alkaline phosphatase may also add an avidity factor to the affinity of the scFv-AP/S fusion protein for the antigen. Moreover, production of MAbs in *E. coli* offers several important features for the standardization of detection assays. Production of scFv-enzyme fusion proteins might give a better reproducibility of the detection of antigens in various immunoassays since the highly variable step of antibody enzyme conjugation is omitted. The recombinant scFv antibodies can be purified via co-expressed affinity tags [2,16] to yield ultra pure, ready to use alkaline phosphatase conjugated reagents.

It is interesting that all the scFv expressing clones isolated with the phage display procedure are BNYVV-specific and do not react with antigens from the *C. quinoa* homogenate. Phage display rather allows PhAbs to be selected on the presence of a specific epitope than on the basis of immunogenicity. For example, glycoproteins are highly immunogenic. Therefore, the immune response elicited by a virus preparation that is slightly contaminated with a plant glycoprotein will be biased to that particular glycoprotein. The result is a high proportion of host-specific MAbs. If the same virus preparation would be used to select PhAbs from a combinatorial antibody library, specific clones are obtained against the most abundant epitope present; i.e., the virus. This was what was observed for the BNYVV virus preparation, the virus is almost pure but still contains some plant contaminants, yet the PhAbs obtained with this method are BNYVV-specific. It would be worthwhile to evaluate their use in a routinely applied standardized detection assay for BNYVV in sugar beets. They certainly have potential, as they are highly BNYVV-specific.

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Chapter 4

pSKAP/S: an expression vector for the production of single-chain Fv alkaline phosphatase fusion proteins

ABSTRACT

The vector pSKAP/S, was constructed to enable overexpression of single-chain variable fragment antibody (scFv)-alkaline phosphatase fusion proteins. In pSKAP/S, the scFv were genetically fused to the mutated *Escherichia coli* PhoA/S gene that encodes an alkaline phosphatase with increased specific activity. The restriction sites incorporated into pSKAP/S allowed the scFv genes to be easily transferred from pUC119-derived phagemid vectors, that are used frequently in phage display antibody library technology. Strong transcriptional control of expression was achieved using the tetracycline promoter, and induction of different individual clones with anhydrotetracycline resulted in secretion of most of the scFv-alkaline phosphatase fusion proteins into the culture medium. Although some of the clones secreted fusion proteins that were retained in the periplasm, these proteins could be isolated with a simple extraction procedure. Increased amounts of a scFv-alkaline phosphatase fusion protein were obtained when expressed in the pSKAP/S vector compared with expression in a vector incorporating the *lac* promoter. Testing for binding of the scFv-alkaline phosphatase fusion proteins to antigen was possible in an ELISA without the need for additional enzyme-conjugated antibodies. The pSKAP/S vector was successfully used to obtain scFv fragments from a preparation of phage-antibody clones after sub-cloning and expression of individual clones as scFv-alkaline phosphatase fusions, whereas fewer clones (and clones with different properties) were obtained from the same phage-antibody preparations when expressed as soluble scFv fragments. Therefore, the pSKAP/S vector was shown to be useful in extending the range of scFv obtained from phage display libraries.

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INTRODUCTION

Recombinant antibodies with unique binding specificities can be selected from large combinatorial phage display single-chain variable fragment (scFv) antibody libraries. The phage-antibodies are selected by successive rounds of binding to antigen, elution and growth of the eluted phage in *Escherichia coli* without the need for animal immunization [6,9,20,22,27]. Usually, binding of recombinant antibodies to antigen is detected by means of linked peptide-tags (for example, c-myc [9], Flag [10], E-tag, Pharmacia). However, sometimes inconsistencies are observed upon analysis of the soluble scFv obtained. For example, soluble scFv-c-myc antibodies, selected against beet necrotic yellow vein virus (BNYVV: Chapter 3) and potato leafroll virus (PLRV), failed to bind to their respective antigens (Unpublished results). Yet, various scFvs, derived from the same selection process, bound specifically to their antigens when expressed on filamentous phage.

Single-chain Fv comprise the variable domains of the heavy and light chains of immunoglobulin molecules connected by a short peptide sequence (c. 10-15 amino acid residues). It may be that some scFv-c-myc fusion proteins do not fold properly [21] because the hydrophobic residues of the variable/constant domain interface, that are normally shielded in the whole antibody molecule by the constant domains of the heavy and light chains, are exposed in the scFv molecule. Fusion of scFv to the pIII coat protein of filamentous phage may mimic the shielding effect of the hydrophobic residues. In addition, several scFvs may be expressed on a single phage particle, thereby increasing the avidity of binding to antigen, in contrast to the monomeric scFv-c-myc.

The expression of scFv-alkaline phosphatase (scFv-AP) fusion proteins promotes the formation of bivalent antibodies, due to the dimeric nature of active alkaline phosphatase (AP., [1,4,7,12,13,15,26,28,29]). Moreover, the use of an improved alkaline phosphatase enzyme (AP/S) with a reported 35-fold higher specific activity [18] compared to the *E. coli* wild-type protein, increases the absorbance values obtained in ELISA [12].

In addition, increased levels of expression of scFv are possible through improvements to the expression vector. Although the *lac* promoter is used frequently, it is known to be leaky, and cell lysis often occurs prior to induction of protein expression by IPTG [24]. In contrast, the *Tet*A tetracycline promoter/operator of the Tn10 Tc^R gene (reviewed by Hillen and Berens, [8]) is strongly controlled and has been used to express antibody fragments in *E. coli* [25]. Therefore, the vector pSKAP/S was constructed to enable strongly regulated expression of scFv-AP/S fusion proteins, under the transcriptional control of the *Tet*A promoter in *E. coli*. The results presented in this paper demonstrate the utility of application of pSKAP/S to the expression of a variety of scFv.

MATERIALS AND METHODS

Bacterial strains

E. coli strains used for the expression of scFv-AP/S fusion proteins were TG1 (K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*D5/F'*tra*D36, *pro*A$^+$B$^+$, *lacI*^{q}, *lacZ*ΔM15) and XL1-Blue MRF' Kan (Stratagene) (K12, Δ(*mcrA*)183, Δ(mcrCB-hsdSMR-mrr)173, *endA*1 *Sup*E44 *thi*-1 *recA*1 *gyrA*96 *relA*1, *lac* F' *pro*AB *lacI*^{q}ZΔM15 Tn5 (Kan^r)).

A

```
.....pSKAP/S.....
tct agt aag ctt gca tgc aaa ttc tat ttc aag gag
      HindIII
...../.....PEL-B leader.....
          M   K   Y   L   L   P   T   A   A
aca gtc ata ATG AAA TAC CTA TTG CCT ACG GCA GCC

.....
 A   G   L   L   L   L   A   A   Q   P   A   M
GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG
                      SfiI

.../.....Polylinker...../...Spacer..
 A   Q   V   Q   L   Q   A   R   L   A   A   A
GCC CAG GTG CAG CTG CAG GCG CGC CTG GCG GCC GCA
              PstI    AscI        NotI

.../.....Alkaline phosphatase...../.....
 A   R   A   P       L   G   D   I   A   H   H
GCC CGG GCA CCA.....CTG GGG GAT ATC GCA CAC CAT
  XmaI                      EcoRV

.....His-tag../STOP/.....pSKAP/S.....
 H   H   H   H   -
CAC CAT CAC CAT TAA GGA TCC gaa ttc tga gct tga
                  BamHI
```

B

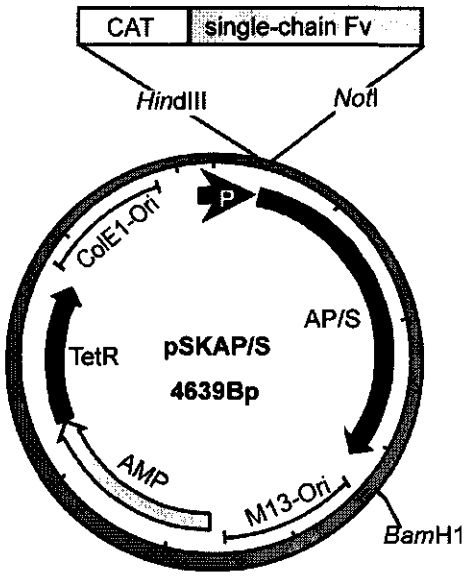

Figure 4.1. **A**) Genetic organization, and sequence of the multiple cloning site of pSKAP/S. **B**) The arrows show the position of the *Tet*A promoter (P) and the genes encoding mutated alkaline phosphatase (AP/S), ampicillin resistance (AMP^R) and the tetracycline repressor (*Tet*R). The restriction sites that were used for the construction of this vector are shown and the insertion sites for chloramphenicol acetyltransferase signal peptide (CAT) and scFv encoding DNA fragments are indicated.

Construction of pSKAP/S

The adapters ASK51 (5'-CTA GTA AGC TTC TAG AGG ATC CGA ATT CTG-3') and ASK52 (5'-AGC TCA GAA TTC GGA TCC TCT AGA AGC TTA-3') were mixed to introduce a new multiple cloning site into pASK75 [25]. After heating for 10 min at 94°C, they were cooled slowly to room temperature and ligated (10:1 molar ratio) into the *Xba*I and *Hin*dIII digested vector fragment of pASK75. This removed the *Xba*I and the *Hin*dIII sites from pASK75 and introduced a new multiple cloning site (Fig. 4.1). The resulting vector (pSK) was transfected to *E. coli* XL1-blue MRF' Kan bacteria (Stratagene). The purified pSK

vector was isolated from pooled transformed bacteria and digested with *Hin*dIII and *Bam*HI. The optimized PelB-AP/S fragment [12] was *Hin*dIII/*Bam*HI digested, ligated into pSK, transfected to *E. coli* XL1-blue MRF' Kan bacteria and grown overnight at 37°C on selective agarose plates (containing 100 µg AMP/ml LB agarose).

Induction of expression in pSKAP/S

Individual transformants were picked and grown overnight at 28°C in LB medium (100 µg AMP/ml). Prior to induction the bacteria were diluted 1:100 in 2TY medium (100 µg AMP/ml) and grown at 37°C. When the cultures had reached late log-phase, the temperature was decreased to 16°C for 1 h. Then anhydrotetracycline (aTc) was added (0.2 µg/ml final concentration) and the bacteria were grown for 18 additional hours and tested for alkaline phosphatase activity. For this, 10 µl of the bacterial suspension was added to 200 µl *p*-nitrophenyl phosphate substrate and incubated at room temperature.

Cloning of scFv into pSKAP/S

Preparations of phage-antibodies (from a combinatorial library) were obtained after four rounds of selection on antigen were used to infect *E. coli* XL1-blue MRF' Kan bacteria (Stratagene) which were then plated on selective LB plates (100 µg AMP/ml) and grown overnight. The plasmids were purified (Midiprep, Promega) digested with *Hin*dIII and *Not*I and the scFv-encoding DNA fragments were ligated into *Hin*dIII/*Not*I digested pSKAP/S vector and transfected to *E. coli* XL1-blue MRF' Kan bacteria. Clones expressing anti-PLRV scFv were expressed in *E. coli* TG1 cells. Transformed bacteria were picked and tested for the expression of scFv-AP/S fusion proteins as described above.

Purification of scFv-AP/S fusion proteins

Bacteria were pelleted and then resuspended in 1/20 of the original culture volume, of 50 mM Tris/HCl pH 8 buffer, containing 30% sucrose and 1 mM EDTA. After incubation for 5 min at 0°C, the bacteria were pelleted and subjected to osmotic shock by the addition of 1/20 the original culture volume of 5 mM $MgSO_4$ and incubated for 45 min at 0°C. The bacterial debris was pelleted and residual EDTA was removed from the resultant soluble periplasmic extract through a buffer exchange, using a G25 gel permeation column (Pharmacia). The $(His)_6$-tagged scFv-AP/S fusion proteins were purified with immobilized metal affinity chromatography (IMAC) using Zn^{2+} charged chelating Sepharose (Pharmacia, Uppsala, Sweden) according to Lindner et al [16]. Bound proteins were eluted with PBS containing 250 mM imidazole and 1 M NaCl.

ELISA

An indirect ELISA was used to assess the specificity of the scFv-AP/S fusion proteins according to standard methods. Briefly, the wells of a 96-well microtiter plate were coated

with the appropriate antigen (2 µg/ml in 0.1 M Na_2CO_3) for 2 h at 37°C. After blocking with PBTM-5% (PBS containing 0.1% Tween-20 and 5% skimmed milk powder) at 200 µl/well for 30 min at 37°C, the wells were incubated with scFv-AP/S diluted in 100 µl of PBTM-1% for 1 h at 37°C. The reaction was visualized by incubation with *p*-nitrophenyl phosphate. For PLRV, this was done as previously described [7].

BIAcore measurements

Binding of isolated scFv-AP/S fusion proteins to lipopolysaccharides (LPS) of *Ralstonia solanacearum* was analyzed using a BIAcore 2000 (Pharmacia Biosensor [11]). Streptavidin (10 µg/ml in 10 mM sodium acetate buffer of pH 4.6) was immobilized using the amine coupling kit from the manufacturer with a contact time of 10 min at a flow rate of 10 µl/min, yielding about 1100 response units (RU). The microsensor chip was then incubated with biotinylated LPS (biotinylated with LC-biotinhydrazide according to the protocol of the manufacturer, Pierce) until saturation was observed (5000 RU). For measurement of the association kinetics, 10 µl of purified scFv-AP/S (0.2 µg scFv-AP/S per ml) was applied at 10 µl/min. Dissociation kinetics were observed for three minutes. Between each measurement the microsensor chip was washed subsequently for 30 s with NaOH (100mM; 10 µl/min) and 30 s with HCl (100 mM; 10 µl/min) for regeneration.

RESULTS

To construct pSKAP/S, the alkaline phosphatase gene containing a mutation that enhances the specific activity of the enzyme 35-fold, was recloned into the vector pSK (Fig. 4.1). The resulting pSKAP/S vector that contains a tetracycline-inducible promoter/operator region was used to express AP/S on induction with different amounts of aTc. ATc is a tetracycline analogue that can induce protein expression more strongly than tetracycline although it has less antibiotic activity. The *Tet*A-promoter was fully derepressed on addition of 100 ng aTc ml^{-1} (Fig. 4.2A). The scFv BNY10, specific for BNYVV, could not be expressed as a functional scFv-c-myc fusion protein. However, after subcloning and expression into pSKAP/S, functional BNY10-AP/S fusion proteins that detected BNYVV in ELISA were produced (Fig. 4.2B). A direct comparison was made of the amounts of murine hybridoma-derived BNYVV-AP/S fusion protein [12] obtained after expression in the vectors pDAP2/S [13] and pSKAP/S. The relative amounts of the scFv-alkaline phosphatase fusion proteins expressed were compared by measuring alkaline phosphatase activity in the bacterial culture supernatant fluids. Expression levels were greater in cells harboring pSKAP/S in both TG1 and XL1-Blue MRF' bacterial strains (Fig. 4.2C). In addition, secretion of several other scFv-AP/S fusion proteins into the periplasm was also higher in the XL1-blue MRF' *E. coli* cells than in TG1 *E. coli* cells (data not shown).

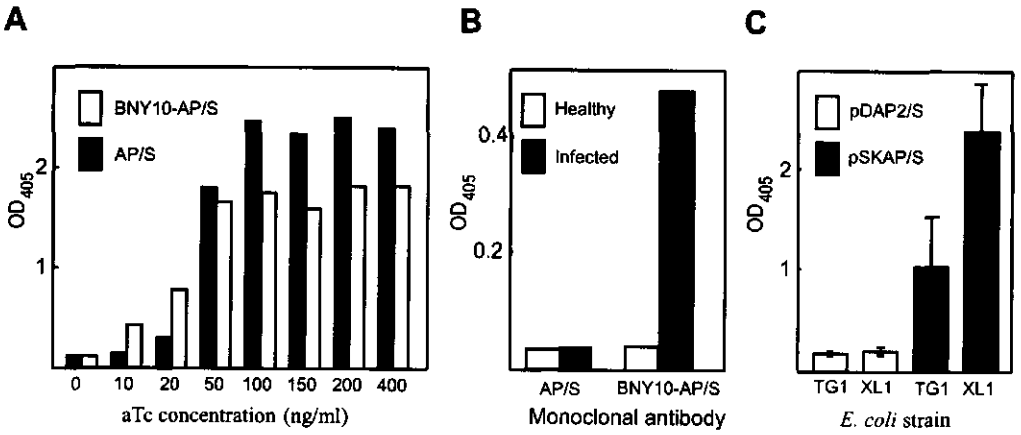

Figure 4.2. A) Induction of pSKAP/S with various amounts of anhydrotetracycline (aTc). The pSKAP/S vector produces either non-fused alkaline phosphatase (AP/S) or a fusion protein of AP/S with a single-chain variable fragment (BNY10-AP/S) directed against beet necrotic yellow vein virus (BNYVV). B) ELISA for testing of AP/S and BNY10-AP/S using either healthy or BNYVV-infected plant extracts for coating microtiter plates. C) Comparison of the expression (as judged by alkaline phophatase activity within the supernatant) of a murine scFv-AP/S fusion protein in the expression vectors pSKAP/S and pDAP2/S and the influence of the *E. coli* TG1 and XL1-Blue MRF' Kan strains which were used for overexpression.

Table 4.1. Reactivity (number and percentage) of single-chain variable fragments (scFv) derived from a selection against potato leafroll virus (PLRV). ScFv were expressed on phage, as scFv-c-myc or as scFv-alkaline phosphatase fusion proteins (scFv-AP/S).

scFv expression	Total number	Number PLRV-reactive	Percentage PLRV-reactive
ScFv-Phage	48	48	100
ScFv-c-Myc	96	0	0
ScFv-AP/S	48	46	95

The advantages of pSKAP/S for the expression of antibody fragments were demonstrated with scFv selected against PLRV by antibody phage display technology (Table 4.1). ScFv from the fourth and fifth rounds of selection against antigen were expressed on the surface of phage and a high percentage reacted specifically with PLRV in phage-ELISA (Table 4.1). However, when scFv from this selection were expressed as soluble scFv-c-myc fusion proteins, no reaction with PLRV was obtained (Table 4.1). In contrast, when scFv from the fourth round of selection were recloned into pSKAP/S and expressed as scFv-alkaline phosphatase fusion proteins, a high proportion detected PLRV in ELISA (Table 4.1).

Similar results were also obtained after overexpression of *Ralstonia solanacearum* specific scFv in pSKAP/S. Only four scFvs were isolated [5] on screening for scFv-c-myc fusion proteins binding to *R. solanacearum*, amongst which was the scFv designated anti-LPS12. The same phage preparation used to isolate the soluble scFvs was then recloned into pSKAP/S, and a further ten different *R. solanacearum*-binding scFv were isolated (data not shown). In some clones scFv-AP/S fusion proteins were found mainly in the periplasm whereas others were mainly secreted into the culture fluid. This was determined by measuring

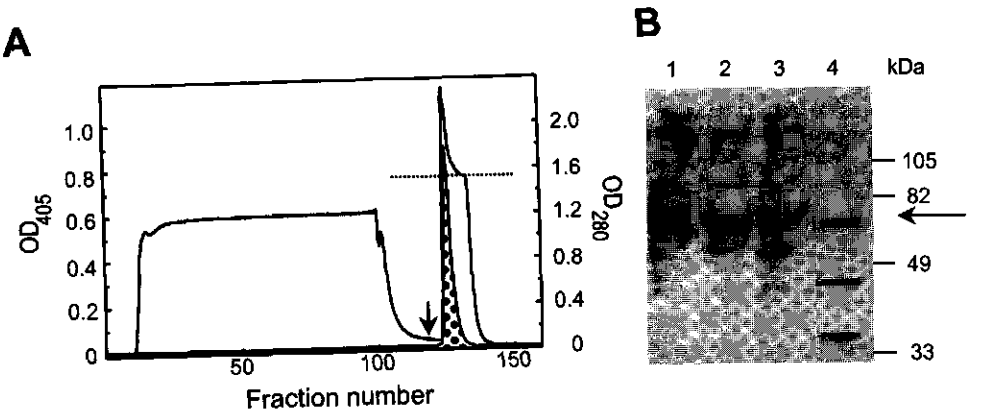

Figure 4.3. **A**) The scFv-AP/S proteins were purified using IMAC and the chromatogram is shown for the purification of anti-LPS7-AP/S (OD_{280}). The arrow indicates the starting point of the elution with imidazole and the background (imidazole only) is indicated by the dotted line. Collected fractions were assayed for AP activity (OD_{405}) as indicated by the shaded area. **B**) The purified scFv-AP/S proteins were analyzed using SDS-PAGE and Coomassie brilliant blue staining: whole cell lysate anti-LPS12-AP/S (lane 1), IMAC purified anti-LPS12-AP/S (lane 2), whole cell lysate anti-LPS7-AP/S (lane 3) and IMAC purified anti-LPS7-AP/S (lane 4). The arrow indicates the position of the scFv-AP/S fusion-protein.

the AP activity in the different cell extracts. The scFv-AP/S fusion proteins were purified from the osmotic shock fraction using IMAC (Fig. 4.3A) and the apparent molecular mass was determined by SDS-PAGE (Fig. 4.3B). In the purified fraction, a major band of the expected 75 kDa was present and a yield of approximately 2.5 mg per liter of culture was obtained. This was also true for the newly isolated scFv, LPS7-AP/S, which was seen to be partially degraded. Despite this partial degradation it showed improved association kinetics when comparable amounts of the scFv anti-LPS7-AP/S and anti-LPS12-AP/S fusion proteins were applied in Biacore analysis (Fig. 4.4). Anti-LPS12-AP/S fusion proteins dissociated more slowly from the LPS-matrix than anti-LPS7-AP/S fusion protein. The non-fused AP/S protein did not bind to LPS, and fusion proteins, like anti-LPS33-AP/S which had fast rate of dissociation, would probably not have been isolated without the use of the vector pSKAP/S. This emphasizes the need for optimal expression systems for soluble scFv.

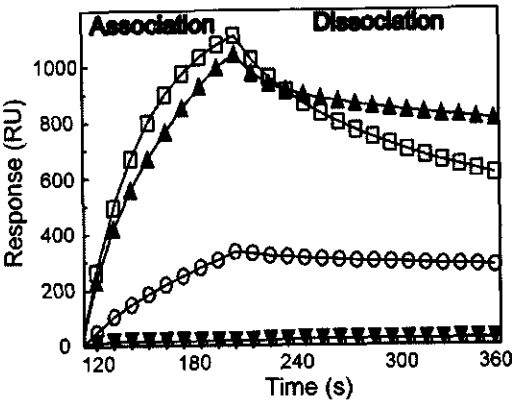

Figure 4.4. Biacore sensorgram of the scFv-AP/S fusion proteins anti-LPS7-AP/S (▲), anti-LPS12-AP/S (○), anti-LPS33-AP/S (❑) and AP/S (▼). Binding of scFv-AP/S to lipopolysaccharides on the chip (association) was monitored for 200 s. Then buffer was applied to the chip and the release of the fusion proteins (dissociation) was observed for a further three minutes.

DISCUSSION

The superiority of the pSKAP/S vector over previously described expression systems [12,13] was demonstrated by the overexpression of various scFv-AP/S fusion proteins, several of which could not be expressed as soluble scFv-c-myc fusion proteins. Moreover, convenient restriction sites allow the simple transfer of scFv genes from vectors commonly used in phage display like pHEN and the pCANTAB series [9,22,27]. Expression in pSKAP/S produced functional scFv-AP/S fusion proteins that were either secreted into the culture fluid or were retained in the periplasmic space. Screening of scFv-AP/S fusion proteins for antigen binding was possible by a simple ELISA without the need for additional enzyme-conjugated antibodies.

In contrast to the commonly used IPTG-inducible *lac* promoter, the *Tet*A promoter allows for induction of expression independently of the metabolic state of the bacteria. Even in the presence of high initial glucose concentrations, the *Lac* promoter becomes leaky due to glucose depletion at high cell densities, and the resultant heterologous gene expression causes toxicity and cell lysis prior to induction [24]. Levels of scFv-AP/S expression were found to be higher when the same scFv-AP/S sequence was expressed in the *Tet*A promoter-based vector (pSKAP/S) compared to the *lac* promoter-based vector (pDAP2/S). Bacteria harboring pSKAP/S appear to suffer less from plasmid loss and showed no formation of satellite colonies on ampicillin-containing plates, or lysis of overnight cultures when grown at 37°C. Moreover, induction with aTc is an economical alternative to IPTG as it is used at low concentrations. When the Tet repressor gene was localized on the high copy expression vector pSKAP/S, the amount of 50-100 ng aTc per ml (Fig. 4.2A) was required for full induction of the *Tet*A promoter. This, while only 20 ng aTc per ml was required in case the Tet repressor gene was localized on the *E. coli* chromosome [17]. Obviously, stronger repression is obtained when the Tet repressor gene is located on a high copy number plasmid than when it is integrated into the *E. coli* chromosome. However, there were no apparent adverse effects from the high levels of Tet repressor proteins, although they have been reported to be toxic when expressed at high levels. [17,25].

The vector pSKAP/S was shown to be a useful tool for the overexpression of scFv-AP/S fusion proteins. Also, it may usefully be applied to express other proteins that are toxic to *E. coli*, for example, restriction endonuclease *CFr*9I [17]. Moreover, incorporation of the *Tet*A promoter may be of general advantage in the construction of combinatorial antibody libraries because the *Tet*A promoter/operator can be gradually induced over a wide range of aTc concentrations. Bacteria that produce toxic scFv have a growth disadvantage compared to bacteria expressing less toxic scFvs, and the former will tend to be outgrown. If measures are not taken to prevent expression of the scFvs, prior to rescue with helper phage, this may result in the desired antibodies being lost from the antibody pool.

From the selected scFv-AP/S fusion proteins produced in *E. coli*, several showed partial degradation when analyzed by SDS-PAGE, for example, LPS7-AP/S (Fig. 4.3B). Degradation is frequently observed on expression of foreign genes in *E. coli* and may be the result of the formation of inclusion bodies in the cytoplasm, or aggregates in the periplasm (both are known to induce the expression of proteases). It is possible that human antibody sequences may not be recognized efficiently by the folding machinery in *E. coli* cells. This may result in the improper folding of the scFv and the subsequent formation of inclusion bodies. The bacterial strains used for expression may be influential and indeed we observed higher levels of secretion with the XL1-blue MRF' strain, as previously reported [2]. However, others have shown expression of Fab fragments to be independent of the bacterial strain [25]. Nevertheless, expression of scFv-AP/S fusion proteins with the aid of pSKAP/S in XL1-blue MRF' bacteria allowed several specific scFv to be isolated and characterized. These will now be further developed into higher affinity, stable immunoassay reagents, for example by site directed mutagenesis [21] or chain shuffling [19].

The correct folding of scFv has been shown to be dependent on the presence of some key amino acid residues [21,14]. In addition, Freund and co-workers [3] have shown the existence of an intermediate folding state between the correct and the misfolded states of scFv in the periplasm. Fusion of scFv to *E. coli* AP/S may cause the heterologous protein to be recognized by the chaperonins of *E. coli*, and furthermore, it may stabilize the scFv-AP/S fusion protein by shielding the hydrophobic residues on the surface of the scFv, thereby allowing a higher proportion of the expressed scFvs to fold properly. As the construction of all scFvs leads to the exposure of the residues of the former variable/constant domain interface, this strategy might lead to a generally applicable method for the expression of scFv fragments.

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Chapter 5

Application of phage display in selecting tomato spotted wilt virus-specific single-chain Fvs for sensitive diagnosis in ELISA

ABSTRACT

A panel of recombinant single-chain antibodies (scFvs) against structural proteins of tomato spotted wilt virus was retrieved from a human combinatorial scFv antibody library using the novel phage display technique. After subcloning the encoding DNA sequences in the expression vector pSKAP/S, which allowed the scFvs to be expressed as alkaline phosphatase fusion proteins, seventeen different scFv antibodies were obtained. Twelve of these scFvs were directed against the nucleoprotein (N) and five against the glycoproteins (G1/G2). Five of the N-specific antibodies cross-reacted with two other tospoviruses TCSV and GRSV, but none recognized the more distantly related tospoviruses INSV, WSMV, IYSV or PSMV. The successful use of one of the retrieved antibodies as coating and detection reagent in a double antibody sandwich ELISA showed the potential of the phage display system in obtaining antibodies for routine TSWV diagnosis.

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INTRODUCTION

Tomato spotted wilt virus (TSWV), the representative of the *Tospovirus* genus within the family *Bunyaviridae*, is the causal agent [28] of a major viral disease in many crops. With an estimated annual crop loss of over one billion US $, TSWV ranks among the top-ten of most detrimental plant viruses worldwide [8,23]. The spread of this plant pathogen is difficult to control, for it has a wide host range of over 1000 plant species including mono- and dicotyledons of more than 80 different families. Tospoviruses are transmitted in a persistent manner by thrips, which over the recent years have become resistant against most applied insecticides. The spherical tospoviral particle (70-110 nm in diameter) contains three structural proteins: a 28 kDa nucleoprotein (N) which is tightly associated with three genomic RNA segments and two glycoproteins of 78 kDa (G1) and 58 kDa (G2) which are associated with the viral envelope. Furthermore low amounts (10-20 copies per virion) of the viral polymerase (331 kDa) are present in the virus particle.

Hardly any durable resistance genes against tospoviruses are available for conventional breeding purposes [23]. TSWV resistance has been obtained by transgenic expression of the nucleoprotein or movement protein (NS_M) gene sequences of TSWV into tobacco [24] and tomato plants [33]. However, the use of transgenic plants for production of crops has been limited to date and epidemics of tospoviruses are still prevented by sanitary measures in combination with surveillance of disease development. Therefore, to prevent the spread of tospoviruses, diagnostic assays like ELISA or PCR have to be developed which are suitable for rapid detection of infected plants and, moreover, can be used for certification of plant propagation materials.

High quality polyclonal antisera have been raised against several established members of the genus *Tospovirus* (Table 5.1) including TSWV [7], groundnut ringspot virus (GRSV) [5], impatiens necrotic spot virus (INSV) [20], tomato chlorotic spot virus (TCSV) [5] and watermelon silverleaf mottle virus (WSMV) [36]. They are now widely applied for tospovirus detection and with the aid of a double antibody sandwich (DAS)-ELISA [9, 35] they form a good compromise between sensitivity, ease and expense of application. However, the applied polyclonal antisera are generally based on N-specific antibodies and recognize mainly the species against which they were raised. Thus far, members of the genus *Tospovirus* have been classified into four distinct serogroups [1,6] (Table 5.1) which are a direct reflection of the sequence homologies (50-80%) of the N protein within the different tospovirus species [5]. The glycoproteins, on the other hand, are more conserved as they share common epitopes, and polyclonal antibodies raised against the glycoproteins of one virus species also detect others [2]. However, as the N protein is by far the most abundant viral protein in infected plants and tospoviruses moreover tend to lose their envelop during several passages in plants [25], this protein remains the target of interest for serological detection purposes.

Table 5.1. Members of the genus tospovirus used in this study.

Species	Abbreviation	Sero-	
		group	type
Tomato spotted wilt virus	TSWV	I	
Tomato chlorotic spot virus	TCSV	II	I
Groundnut ringspot virus	GRSV	II	II
Impatiens necrotic spot virus	INSV-NL	III	
	INSV-US		
Watermelon silverleaf mottle virus	WSMV	IV	
Iris yellow spot virus	IYSV	New	
Physalis severe mottle virus	PSMV	New	

Polyclonal antisera, although widely applied in routine diagnosis, are available in limited amounts and the specificity varies from batch to batch. Therefore, they are increasingly replaced by monoclonal antibodies (MAbs), as these are defined reagents that can be produced indefinitely [19]. In contrast to polyclonal antibodies, MAbs recognize single epitopes and may allow escape of viruses that lack those epitopes. To guarantee durable recognition of TSWV in the future, antibodies against conserved epitopes are required. Several MAbs have been raised against TSWV [1,16,26,29]. However, as new serogroups are still being established (Table 5.1), e.g. iris yellow spot virus (IYSV) [4] and physalis severe mottle virus (PSMV) [Kormelink, unpublished], there is a need for expansion of the available panel of MAbs that can be used for serological detection.

Recombinant antibody technology in combination with the novel phage display technology [15, 22] (Fig. 1.5) provides an extremely useful approach for the production of target-specific antibodies [13,34]. Thus far, highly specific antibodies have already been obtained in this way against several important plant pathogens [11,12,14,30,37] without the use of laboratory animals and time-consuming immunization protocols.

In this report we describe the use of the phage display technique to raise a broad panel of TSWV-specific recombinant antibodies from a human combinatorial antibody library [34]. Separate selections were carried out to retrieve recombinant scFvs against the major structural proteins of TSWV, i.e. the N protein and the G1 and G2 glycoproteins. As G1 and G2 form heterodimers that are hard to separate without introduction of a denaturating step, PhAbs were selected to either of these proteins. The scFv encoding genes retrieved were expressed in *E. coli* as ready to use antibody-enzyme fusion proteins [10] which were used for characterization of their reaction with TSWV and several other tospoviruses. In addition, one of the selected monoclonal scFvs was compared to a TSWV-specific polyclonal rabbit antiserum for its use as a coating and detecting reagent in a DAS-ELISA.

MATERIALS AND METHODS

Bacterial strains

E. coli strain TG1 was used for the panning experiments. Expression of scFv-AP/S fusion proteins was performed in XL1-Blue MRF' Kan bacteria (Stratagene).

Purification of tomato spotted wilt virus

Infectious virus particles of TSWV Brazilian strain (BR-01) were isolated from systematically infected *Nicotiana rustica* var. America leaves according to Gonsalves and Trujillo [9]. Purified virions were finally collected from a 20-40% sucrose gradient, subsequently pelleted, resuspended in water and stored at -70°C. Concentration of viral protein was measured using the Biorad protein assay (Biorad).

Purification of viral nucleocapsids

Nicotiana rustica var. America plants were inoculated with TSWV (BR-01). After 8-18 days, systemically infected leaves were harvested and homogenized in 4 ml/g TAS-E buffer (freshly prepared: 0.1 M Tris-HCl; 0.01 M EDTA; 0.01 M Na_2SO_3; 0.1% Cysteine, pH 8.0). The homogenate was filtered through 2 layers of cheesecloth and put on ice. The supernatant was centrifuged for 10 min at 3,000 rpm at 4°C in a GSA rotor, subsequently transferred to Ti45 tubes and centrifuged a second time for 30 min at 20,000 rpm at 4°C in a Ti45 rotor. The pellet obtained, containing viral nucleocapsids, was resuspended in 0.4 ml/g TAS-R buffer (freshly prepared: 0.01 M Tris-HCl; 0.01 M EDTA; 0.01 M Na_2SO_3; 0.1% Cysteine; 0.01 M Glycine; 1% Nonidet P40, pH 7.9) while gently stirring for 1-2 h at 4°C. A volume of 10 ml of the nucleocapsid material was enlarged to 30 ml using TAS-R buffer and layered on a 30 ml 30% sucrose cushion (30% w/v sucrose in TAS-R buffer). After centrifuging for 2.5 h at 28,000 rpm at 4°C in a Ti45 rotor, the supernatant was decanted, the nucleocapsid pellet resuspended overnight in PBS and stored at -70°C until further use. The concentration of N protein was measured using the Biorad protein assay.

Preparation of virus-infected plant material for ELISA-assays

Approximately 2 weeks after inoculation of *Nicotiana benthamiana* plants with tospoviruses GRSV (SA-05), INSV (US-01), INSV (NL-07), IYSV, PSMV, TCSV (BR-03), TSWV (BR-01) or WMSV (Table 1), systemically infected leaves were harvested and homogenized 1:10 w/v in extraction buffer (1 g leaf material/10 ml PBS 2% polyvinylpyrolidon) for use in ELISA.

Selection of recombinant antibodies from a combinatorial antibody library

Selection of target specific phage-antibodies (PhAbs, i.e. phages expressing functional scFv on their surface) from the human combinatorial antibody library [34] was performed

according to Griep and co-workers [12] with a few modifications. Immunosorbent tubes (Maxi-sorb, Nunc) were coated with either purified TSWV or with purified nucleocapsids, diluted in 50 mM Na_2CO_3, pH 9.8 for 18 h at 4°C, according to Table 5.2. The derived PhAbs were stored at 4°C until use in the next panning round or in phage-ELISA.

Table 5.2. The concentration of nucleocapsid (N) and complete tomato spotted wilt virus (TSWV) as was used for coating of immunosorbent tubes in combination with the number of washings applied during the panning procedure.

Target	Panning round	Quantity (µg)[a]	Washings
Nucleocapsid	1	200	20
	2	200	20
	3	40	40
	4	4	40
Intact virion	1	400	20
	2	200	20
	3	34	40
	4	4	40

[a]Total amount of target-antigen that was used for coating of the immunosorbent tubes.

Subcloning of scFv encoding genes in expression vector pSKAP/S

After the fourth round of selection the XL1 blue MRF' Kan bacteria were scraped from the plate and the scFv encoding phagemids were extracted using a plasmid DNA purification system (Wizard Midiprep, Promega). Phagemid DNA was digested [27] with *Hin*dIII and *Not*I and the excised scFv-encoding DNA fragments were gel-purified and subsequently ligated into *Hin*dIII/*Not*I digested pSKAP/S vector [10]. The ligation mix was transfected to *E. coli* XL1-blue MRF' Kan bacteria and the cells grown overnight at 37°C under selection pressure of 100 µg ampicillin (Amp) per ml LB agarose [27].

Production and purification of scFv-alkaline phosphatase fusion proteins

Individual transformants were picked and grown overnight at 28°C in LB medium (100 µg Amp/ml). Prior to induction the bacteria were diluted 1:100 in 2TY medium (100 µg Amp/ml + 2% glucose) and grown at 37°C. When the cultures had reached late log-phase (OD_{600} of 0.9) the temperature was decreased to 16°C for 1 h. Subsequently anhydrotetracycline (aTc) was added (0.2 µg/ml final concentration) and the bacteria were grown for an additional 18 h at 16°C. Bacterial cells were pelleted and resuspended in 1/20 volume (referring to the original culture volume) 50 mM Tris/HCl, pH 8 buffer containing 30% sucrose and 1 mM EDTA. After incubation for 5 min at 0°C, the scFv-alkaline phosphatase (scFv-AP/S) fusion proteins were extracted from the periplasm with 1/20 volume (referring to the original culture volume) of 5 mM $MgSO_4$ during 45 min at 0°C. In some cases the scFv-AP/S fusion proteins were further purified, using immobilized metal affinity chromatography (IMAC) according to Lindner and co-workers [21].

_Mva_I-fingerprinting

To analyze the diversity of the recloned scFvs, restriction fragment length polymorphism (RFLP) fingerprinting was performed on PCR amplified scFv-DNA. Single colonies were picked and grown for 4 h in 2TY (100 µg Amp/ml). From these suspensions, 2 µl was taken and added to a 48 µl PCR mix containing 2.5 µM dNTPs; 0.25 U Super Taq DNA polymerase (HT Biotechnology, Cambridge, UK); 10 µM pSK-forward (5'-ACT CTA TCA TTG ATA GAG TTA TTT TAC CAC TCC C-3') and 10 µM pSK-reverse (5'-TTT ATC GCT AAG AGA ATC ACG CAG AGC GGC G-3') primers; 2 mM $MgCl_2$ and 50 mM Tris/HCl, pH 8. Amplification was performed in a thermal cycler (Perkin Elmer), 25 cycles (1 min 94°C; 2 min 72°C) were performed. After PCR-amplification, 20 µl of the mix was digested with 10 U of restriction enzyme *Mva*I (Boehringer) for 18 h at 37 °C. Digestion patterns were analyzed on a 3% FMC Metaphor agarose gel (Epicentre Technologies, Madison, USA).

Phage-ELISA

The phage-ELISA was essentially carried out according to Clark and Adams [3] and to Tijsen [32]. After each incubation step the microtiter plates were washed 4 times with PBST (PBS containing 0.1% (v/v) Tween-20). In brief, ELISA plates (Labstar, Costar, Cambridge, UK) were coated overnight at 4°C with 100 µl/well purified viral nucleocapsids, diluted to 2 µg/ml in 0.1 M Na_2CO_3/pH 9.8. Plates were blocked with 200 µl/well PBSTM-5% (PBST containing 5% skimmed milk) for 30 min at 37°C, washed twice with PBST and subsequently incubated for 1 h at RT with 100 µl phage sample dilutions. nucleoprotein-phage-antibody complexes were detected with monoclonal mouse-anti-M13 antibodies (MAb 12E4, 1 µg/ml in PBSTM-2%), and finally with polyclonal rat-anti-mouse antibodies conjugated to alkaline phosphatase (Jackson Immuno-Research Laboratories, Inc., Westgrove, PA) diluted 1:2500 in PBSTM-2%. The ELISA was developed by adding 100 µl *p*-nitrophenyl phosphate (p-NPP) substrate per well and scored for positive reactions by absorbance reading at 405 nm, usually within 60 min.

Antigen coated plate ELISA

An antigen-coated plate (ACP)-ELISA was used to assess the specificity of the recombinant scFv-antibodies, according to standard methods [3, 32], in which the plates were washed four times with PBST between each incubation step. In brief, ELISA plates were coated with either TSWV virions or with purified nucleocapsids, both diluted to 2 µg/ml in 0.1 M Na_2CO_3. After blocking with PBSTM-5% for 30 min at 37°C, the plates were incubated for 1 h at 37°C with recombinant anti-TSWV scFv alkaline phosphatase fusion proteins, diluted 1:1 in PBTM-4% or with PcA-BR01-AP (alkaline phosphatase conjugated polyclonal Rabbit antibodies, raised against the nucleocapsid of TSWV), diluted 1:2000 in PBSTM-2%. The reactions were visualized by incubation with p-NPP substrate and optical densities were measured at 405 nm with an ELISA-reader.

Double antibody sandwich ELISA

A double antibody sandwich (DAS)-ELISA was used to assess the reactions of the recombinant scFv-antibodies towards other tospoviruses. Between each incubation step the plates were washed four times with PBST. In brief, ELISA plates were coated (100 µl/well) for 18 h at 4°C with polyclonal antibodies, raised against the nucleocapsid of TSWV, TCSV, GRSV, INSV, IYSV or a mix of these, diluted 1:1,000 in 0.1 M Na_2CO_3, pH 9.8. After blocking (200 µl/well) with PBSTM-5% for 30 min at 37°C, the plates were incubated with homogenates of either healthy or systemically infected *N. benthamiana* leaves. The plates were incubated with anti-TSWV scFv alkaline phosphatase conjugates, diluted 1:1 in PBTM-4% (100 µl/well) for 1 h at 37°C. The reactions were visualized by incubation with p-NPP substrate and optical densities were measured at 405 nm with an ELISA-reader.

Western blotting

TSWV proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Reactive groups on the membrane were blocked with PBMT-5%. The reactivity of the scFv towards TSWV glycoproteins was shown by incubation of the Western blot with anti-TSWV scFv alkaline phosphatase conjugates and staining with BCIP/NBT substrate.

RESULTS

Selection of recombinant TSWV-specific antibodies

In order to obtain scFv-antibodies directed against TSWV N protein or G1/G2 proteins, four successive rounds of selection (panning) were carried out against purified TSWV nucleocapsids or purified virions, respectively. While the amount of target antigen for coating was reduced and the washing was intensified during the panning procedure (Table 5.2), the recovery of phage-antibodies (PhAbs) from the immunosorbent tubes showed a strong increase for each subsequent round of selection (Fig. 5.1A). After four rounds of selection, the specificity of the "polyclonal" PhAbs mixture, panned against the nucleocapsid, showed an increasing reactivity towards the nucleoprotein in phage-ELISA (Fig. 5.1B).

Characterization of selected monoclonal scFv antibodies

To allow efficient characterization of individual scFv producing clones, the obtained antibody encoding genes were recloned in expression vector pSKAP/S (Chapter 4, [10]) and expressed as scFv alkaline phosphatase fusion proteins. Upon screening in ACP-ELISA several clones were obtained producing monoclonal scFv-AP/S fusion proteins which bound to TSWV proteins. From the panning, carried out against purified TSWV virions, the obtained scFv-AP/S fusion proteins reacted with complete TSWV particles only and not with purified nucleocapsids and are therefore specific to the glycoproteins. Other scFv-AP/S fusion proteins, all derived from the selection against purified nucleocapsids, reacted with complete

TSWV virions as well as with nucleocapsids and are thus directed against the N protein. Further analysis of the selected scFv encoding genes, using RFLP mapping with restriction enzyme *Mva*I, revealed that five different G-specific monoclonal scFvs and 12 different N-specific monoclonal scFvs were obtained. These were designated according to their target specificity and the number of the clone producing each particular scFv-AP/S fusion protein (Table 5.3). The diversity was confirmed by DNA-sequencing (data not shown).

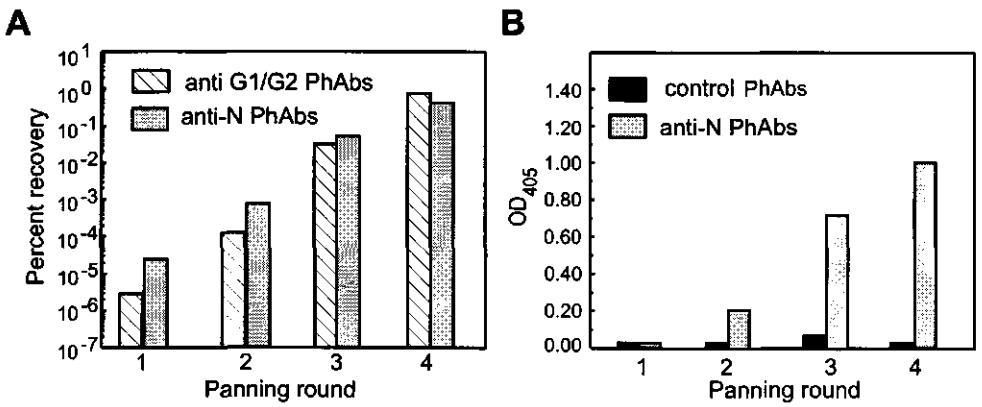

Figure 5.1. Recovery and specificity of TSWV-binding phage-antibodies (PhAbs) in four sequential rounds of panning. PhAbs were applied to either TSWV virion- or N protein coated immunosorbent tubes and allowed to bind. Bound PhAbs were eluted after washing and used to infect *E. coli*. After phage rescue, with helper phage, the PhAbs (anti-TSWV enriched) were used for a new round of panning. **A)** The number of applied and recovered PhAbs was counted and the percentage recovery was plotted for each subsequent panning round. **B)** A phage-ELISA was performed with equal amounts of the selected anti-N-specific PhAbs and compared to comparable amounts of control PhAbs (derived from a panning against another antigen) to show that the binding was N-specific.

Table 5.3. The obtained single-chain Fv-alkaline phosphatase fusion proteins were designated reflecting the target-antigen and the number of the first clone found with a unique *Mva*I restriction pattern.

Target-antigen	
Nucleoprotein (N)	Glycoproteins (G1/G2)
N3-AP/S	G118-AP/S
N13-AP/S	G150-AP/S
N19-AP/S	G180-AP/S
N25-AP/S	G220-AP/S
N28-AP/S	G224-AP/S
N32-AP/S	
N35-AP/S	
N40-AP/S	
N54-AP/S	
N56-AP/S	
N63-AP/S	
N97-AP/S	

Differences in the expression levels were found for the obtained scFv-AP/S fusion proteins. The production in *E. coli* of functional scFv-enzyme fusion proteins varied from less than 50 µg/L of bacterial culture for the scFv-AP/S fusion-protein of N32, to 4 mg /L for G118-AP/S.

All glycoprotein-specific fusion proteins (Table 5.3) reacted in ACP-ELISA with purified TSWV virions. The osmotic shock fraction of G118-AP/S (4 mg/ml) even gave a positive signal when diluted up to 10,000 times (Fig. 5.2). However, when homogenates of TSWV-infected *N. benthamiana* leaves were used for coating in an ACP-ELISA format, only weak reactions were obtained with all G-specific scFv-AP/S fusion proteins. In Western blot analyses, G150-AP/S could be identified as G2-specific and G224-AP/S as G1-specific (data not shown), whereas for the remaining G-binding antibodies the specificity could not be determined as their epitopes were denatured by SDS-treatment.

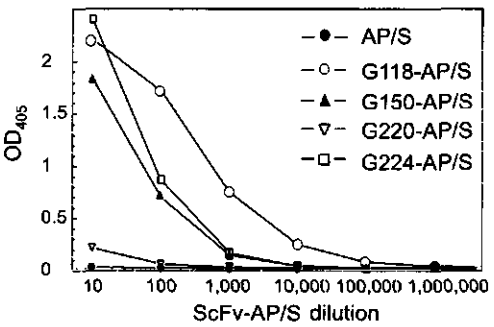

Figure 5.2. Reaction of glycoprotein (G1/G2)-specific single-chain (scFv) alkaline phosphatase (AP/S) fusion proteins in antigen-coated plate ELISA. The G-specific scFv-AP/S fusion proteins were extracted from the periplasm of clones G118, G150, G220 and G224 and applied in serial dilutions into wells of an ELISA-plate coated with TSVW virions. AP/S without a scFv served as a negative control.

Reactivity of TSWV-specific scFvs towards other tospoviruses

In order to analyze whether the selected recombinant antibodies also recognized other tospovirus species besides TSWV, they were tested using homogenates of *N. benthamiana* leaves systematically infected with GRSV, INSV, IYSV, PSMV, TCSV, TSWV or WMSV (Table 5.1). While in an ACP-ELISA no significant reactions were obtained, except for the homologous reaction with TSWV, positive reactions (Fig. 5.3) were observed when aliquots of TCSV-infected plant extract were blotted onto nitrocellulose and N3-AP/S, N19-AP/S and N56-AP/S were used for detection. In addition, the G1-reactive fusion protein, G224-AP/S also reacted in this dot-blot assay, but only with TSWV (data not shown).

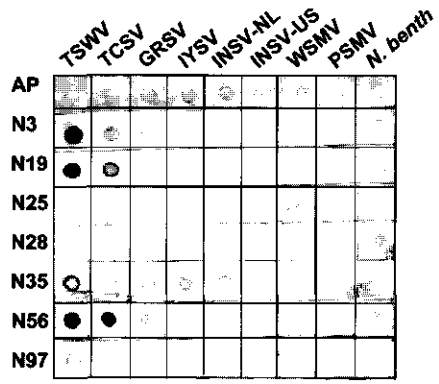

Figure 5.3. Reactivity of the TSWV nucleocapsid-specific single-chain variable fragment alkaline phosphatase fusion proteins in a dot-immunobinding assay to other tospovirus species.

Table 5.4. Results of double antibody sandwich ELISA in which the plates were coated with a polyclonal TSWV N antiserum. Samples containing complete virions (CV), nucleoprotein (N) or buffer (B) were applied and the binding of the various N-specific scFv-AP/S fusion proteins was visualized using p-nitrophenyl phosphate substrate.

	OD_{405} after 2 hours substrate incubation[a]			OD_{405} after 18 hours substrate incubation[a]		
Clone	CV	N	B	CV	N	B
AP/S	0.03	0.03	0.03	0.05	0.05	0.05
3	1.17	1.22	0.03	>3	>3	0.05
13	0.14	0.18	0.03	2.70	>3	0.04
19	1.01	0.96	0.03	>3	>3	0.05
25	0.06	0.07	0.03	1.16	1.6	0.04
28	0.13	0.18	0.03	2.58	2.80	0.12
32	0.04	0.05	0.03	0.43	0.55	0.04
35	0.20	0.55	0.03	>3	>3	0.06
40	0.05	0.07	0.04	0.77	1.14	0.18
54	0.53	0.45	0.03	>3	>3	0.03
56	0.81	0.97	0.03	>3	>3	0.04
63	0.15	0.18	0.03	2.65	2.80	0.04
97	0.08	0.13	0.03	1.58	2.45	0.05

[a]The data were obtained from a representative experiment and the standard deviations (n=2) were smaller than 0.1 for all presented data.

Table 5.5. Results of double antibody sandwich ELISA in which the plates were coated with specific antisera against TSWV N, INSV N, TCSV N or GRSV N proteins. Plant extracts containing the homologous tospoviral nucleoprotein were applied and the binding of the scFv-AP/S fusion proteins was visualized.

	OD_{405} after 2 hours substrate incubation[a]				OD_{405} after 18 hours substrate incubation[a]				
Clone	PE + TSWV	PE + TCSV	PE + GRSV	PE + INSV	PE + TSWV	PE + TCSV	PE + GRSV	PE + INSV	PE
AP/S	0.03[c]	0.03	0.03	0.03	0.06	0.07	0.06	0.05	0.05
3	1.82	0.14	0.04	0.03	>3	2.51	0.30	0.06	0.05
13	0.38	0.03	0.03	0.03	>3	0.16	0.09	0.05	0.12
19	1.30	0.12	0.03	0.03	>3	2.41	0.25	0.06	0.05
25	0.15	0.03	0.03	0.03	2.71	0.56	0.12	0.04	0.04
28	0.37	0.04	0.03	0.03	>3	0.15	0.06	0.06	0.12
32	0.05	0.03	0.03	0.03	0.73	0.05	0.04	0.05	0.04
35	0.81	0.03	0.03	0.03	>3	0.08	0.08	0.06	0.06
40	0.12	0.03	0.04	0.04	2.15	0.07	0.07	0.06	0.18
54	1.11	0.03	0.03	0.03	>3	0.12	0.13	0.12	0.18
56	1.23	0.12	0.03	0.03	>3	2.41	0.36	0.05	0.04
63	0.33	0.03	0.03	0.03	>3	0.06	0.11	0.05	0.04
97	0.22	0.05	0.04	0.03	>3	0.81	0.25	0.05	0.05

[a]The data were obtained from a representative experiment and the standard deviations (n=2) were smaller than 0.1 for all presented data.

In order to determine whether the different results obtained were due to the (ACP-) ELISA format, a DAS-ELISA was performed on TSWV-infected leaf material. To this end, ELISA plates were coated with TSWV-specific polyclonal anti-N serum. Samples of TSWV-infected leaf material, purified TSWV nucleocapsids or TSWV virions were applied and tested for recognition. Results with N-specific scFv-AP/S fusion proteins showed a clear reaction in almost all cases with TSWV containing samples (Table 5.4), although some reactions, e.g. N40-AP/S, showed a lower OD_{405} absorbance, probably due to a lower affinity of this scFv-AP/S protein for the nucleocapsid. The reaction was N-specific, as no reaction above the background was obtained with buffer or when non-conjugated AP/S or G-specific scFv-AP/S fusion proteins were applied.

In analogy to this, a DAS-ELISA was performed in which the plate was coated with each of the N-specific antisera that were raised against the nucleocapsids of six different tospoviruses (Table 5.1). The results from this ELISA (Table 5.5) showed clear reactions with TCSV and GRSV in combination with N3-AP/S, N19-AP/S, N25-AP/S, N56-AP/S and N97-AP/S, although less intensive compared to TSWV. No reactions were observed with any of the more distantly related species INSV, IYSV, PSMV and WMSV.

From the latter results, combined with those obtained in the ACP-ELISA, it is clear that the fusion proteins N3-AP/S, N19-AP/S, N25-AP/S, N56-AP/S and N97-AP/S do show recognition of TSWV, TCSV and GRSV in a DAS-ELISA format but not in ACP-ELISA format. Moreover, whereas GRSV is not recognized in the ACP-ELISA or on dot-blot, this virus is recognized at low efficiency with the aforementioned scFv-AP/S fusion proteins in DAS-ELISA.

Sensitivity in TSWV diagnosis

To test the potential of the derived monoclonal scFv antibodies the "broad-reacting" N56-AP/S fusion protein, able to recognize serogroup I & II species, was purified and further characterized for its applicability in TSWV diagnosis. Different amounts of TSWV-derived nucleocapsids were diluted into healthy plant extracts of *N. benthamiana* and tested in a DAS-ELISA (Table 5.6). At a concentration of 0.1 µg N56-AP/S per ml the detection limit obtained for the N protein was comparable to the detection limit obtained using the polyclonal antibody alkaline phosphatase conjugate at 0.5 µg per ml. This resemblance in sensitivity was also observed when serial dilutions of healthy and TSWV-infected *N. benthamiana* homogenates were screened in a DAS-ELISA. Whereas samples are tested in routine diagnosis at dilutions of 10-100 times, both the scFv-AP/S fusion-protein of N56 (Fig. 5.4A) and the polyclonal antiserum (Fig. 5.4B) detected TSWV in extracts of *N. benthamiana* at dilutions up to 1:16,000.

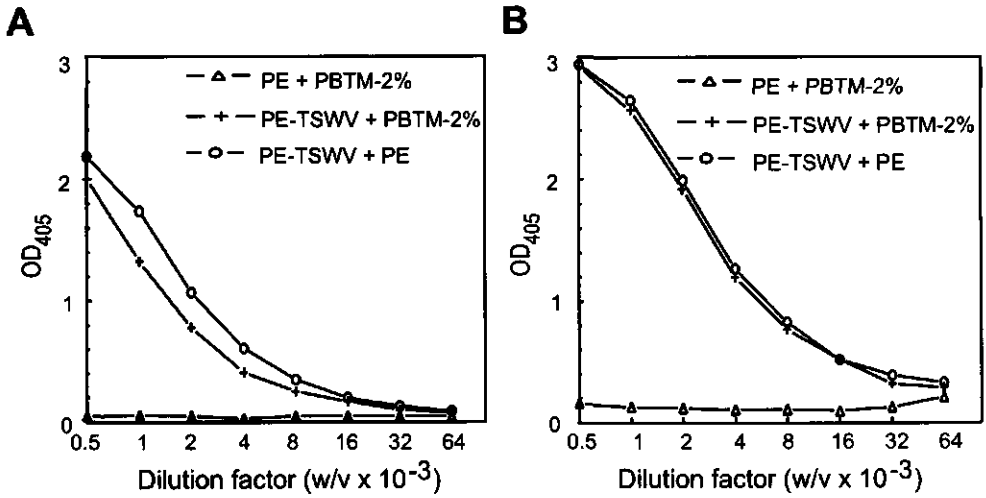

Figure 5.4. A) Determination of double antibody sandwich ELISA detection limit for nucleocapsid protein (N) in TSWV-infected plant extract as assayed **A)** with the single chain variable fragment antibody alkaline phosphatase fusion-protein N56-AP/S or **B)** with a TSWV N-specific polyclonal antiserum conjugated to alkaline phosphatase. The wells of an ELISA plate, coated with a TSWV N-protein specific polyclonal antiserum, were incubated with extracts of healthy *Nicotiana benthamiana* plants (PE) or TSWV-infected *N. benthamiana* plants (PE-TSWV). Serial dilutions, of PE and PE-TSWV, were made either in PE or in PBS containing 2% skimmed milk powder and 0.1% Tween-20 (PBTM-2%). The N-specific polyclonal antiserum AP conjugate was applied at 0.5 µg/ml and the fusion-protein N56-AP/S at 0.1 µg/ml.

Table 5.6. Determination of the detection level for the nucleocapsids of tomato spotted wilt virus using a double antibody sandwich ELISA in which the plates were coated with specific antisera against TSWV nucleocapsids. Compared were the nucleoprotein-specific single-chain N56 fused to alkaline phosphatase (AP/S) and the conventional alkaline phosphatase (AP) conjugated TSWV N-specific polyclonal antibodies. Purified nucleocapsids were diluted in a healthy plant extract (PE).

nucleocapsids (ng/ml)	Antibody-enzyme conjugate	
	N56-AP/S (0.1 µg/ml)[a]	Polyclonal-AP (0.5 µg/ml)[b]
1000	2.31 ± 0,102	2.89 ± 0,015
500	1.98 ± 0,053	2.51 ± 0,049
100	0.77 ± 0,053	1.16 ± 0,087
50	0.40 ± 0,057	0.77 ± 0,041
10	0.14 ± 0,005	0.32 ± 0,026
5	0.08 ± 0,002	0.21 ± 0,030
1	0.06 ± 0,004	0.17 ± 0,004
PE	0.05 ± 0.01	0.14 ± 0,03

[a]ELISA-readings after 2 h incubation with *p*-nitrophenyl phosphate substrate at room temperature + 16 h at 4 C.

[b]ELISA-readings after 2 h incubation with *p*-nitrophenyl phosphate substrate at room temperature

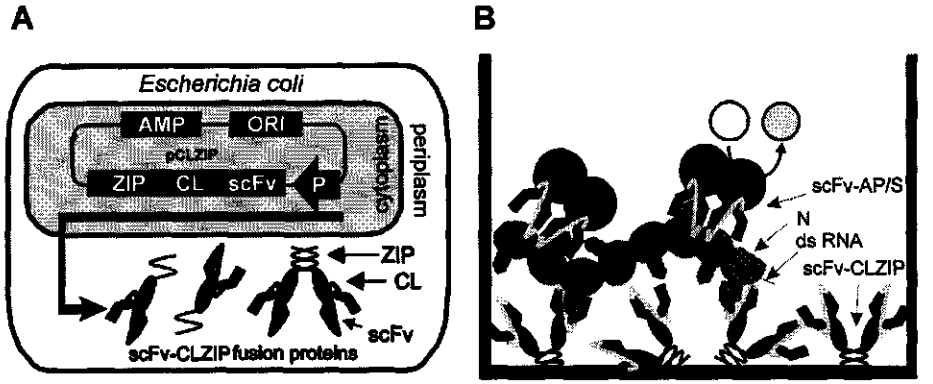

Figure 5.5. A) The alkaline phosphatase gene of N56-AP/S was replaced by the constant Kappa light chain domain (CL) and a leucine zipper domain (ZIP). Transformed bacteria produce fusion proteins of scFv N56 and the CLZIP domains upon induction. The CLZIP-N56 fusion proteins are secreted into the periplasm where they dimerize. **B)** The CLZIP-N56 fusion proteins can be used for coating the wells of an ELISA plate that is subsequently incubated with extracts of TSWV-infected *N. benthamiana* plants (PE-TSWV). For detection of captured nucleocapsids (N) an N-specific scFv alkaline phosphatase fusion protein can be applied (scFv-AP/S).

Development of a complete recombinant DAS-ELISA

While a DAS-ELISA utilizing the TSWV/N-specific polyclonal antiserum as coating reagent and the N56-AP/S fusion protein for detection was highly sensitive for TSWV (Fig. 5.4A), the use of scFv N56 as coating reagent was also evaluated. As scFvs are inactivated upon coating to ELISA-plates [17], a construct of scFv N56 was assembled (Fig. 5.5A) in which the AP/S encoding gene was replaced by a sequence encoding the constant domain of the mouse light-chain (CL), a leucine zipper (ZIP) dimerization domain and a His(6)-tag [18]. The expressed N56-CLZIP protein was purified by IMAC and compared to the polyclonal TSWV/N-specific antiserum for coating efficiency utilizing a DAS-ELISA (Fig. 5.5B). At a coating density of 1 µg/ml the N56-CLZIP fusion protein was equally effective (Table 5.7), which demonstrates that TSWV can be efficiently detected with a DAS-ELISA that is entirely based on recombinant MAbs.

Table 5.7. Comparison of coating and detection efficiency using a combination of conventional TSWV/N specific polyclonal antibodies or recombinant antibodies in a double antibody sandwich ELISA. Healthy plant extract (PE) or TSWV-infected plant extract (PE-TSWV) was applied at 1 g per 20 per ml of extraction buffer.

	Detection reagent					
	PAb-TSWV-AP (0.5 µg/ml)		N56-AP/S (0.1 µ g/ml)		N56-AP/S (1 µg/ml)	
Coating reagent	PE[a]	PE-TSWV[b]	PE[a]	PE-TSWV[b]	PE[a]	PE-TSWV[b]
PAb-TSWV N (0.5 µg/ml)	0.04	0.99	0.03	0.57	0.05	1.39
CLZIP-56 (0.1 µg/ml)	0.04	0.51	0.03	0.36	0.05	0.71
CLZIP-56 (1 µg/ml)	0.04	0.73	0.05	0.71	0.05	1.28

[a]Standard deviations obtained with healthy plant extract (PE) were smaller than 0.006

[b]Standard deviations obtained with TSWV-infected plant extract (PE) were smaller than 0.1

DISCUSSION

With the aid of the phage display system, a series of 17 different TSWV-specific scFvs was retrieved from the Vaughan combinatorial scFv-antibody library [34], utilizing expression as scFv-alkaline phosphatase fusion protein in *E. coli*. Twelve of these scFv-AP/S fusion proteins were identified as N-specific and five were directed against the glycoproteins of TSWV (Table 5.3).

From the series of N-specific scFv-AP/S fusion proteins, N56-AP/S, at a concentration of 0.1 µg/ml could detect as little as 1 ng (Table 5.6) of N protein in a DAS-ELISA. In addition it was shown that the CLZIP fusion protein of scFv N56 can be used as an effective coating reagent (Table 5.7), suggesting that this recombinant scFv represents a promising candidate for development of a complete recombinant diagnostic assay for routine detection of TSWV in plant material.

Extensive ELISA and Western immunoblot analyses have demonstrated that the obtained antibodies recognized at least five epitopes of the three structural TSWV proteins. Fusion proteins G150-AP/S and G224-AP/S were found to recognize continuous epitopes on G2 or G1, respectively, whereas G118-AP/S most likely recognized a discontinuous epitope, as reactivity was absent after mild denaturation of the glycoproteins by SDS-treatment. From the N-specific scFv-AP/S, some reacted with the N-protein of TSWV, whereas others reacted with an epitope that was not only present on the N-protein of TSWV, but also on those of TCSV and GRSV. The N encoding genes of the latter isolates have been sequenced and share around 78% sequence identity [5], but thus far, no scFvs have been observed recognizing a common epitope within the N protein of all different tospovirus species. N56-AP/S is one of few selected MAbs that recognizes up to three different tospoviruses, i.e. TSWV, GRSV and TCSV, and must therefore be directed against a common epitope.

Polyclonal TSWV N-specific antisera, and now also some scFv, have been shown to cross-react with TCSV and GRSV isolates. For a universal tospovirus diagnosis system a scFv is required that recognizes all tospoviruses, even those that currently still escape from detection. However, instead of searching for one single universal scFv, it is recommended to pool a set of broad-reacting MAbs and thus to obtain a specific "polyclonal antiserum" with a defined character and with continuous specificity for all the species within the genus *Tospovirus*.

The data presented in this report have demonstrated the feasibility of the phage display technique to select a large panel of TSWV-specific MAbs and is in strong support for the identification of new series of scFv-antibodies. Some of which likely will have a broader tospoviral detection range. As the phage display technique allows to alternate selections between N (or G1/G2) proteins obtained from different isolates, it may now be possible to guide selection of MAbs towards shared epitopes. After all, selection of MAbs by phage display is not biased to a certain epitope on basis of immunogenicity but merely by the

frequency of occurrence. This means that selection would be for specificity against the most prominent conserved epitopes within the N (or G1/G2) proteins.

Since the tospoviral N protein is involved in many viral processes, such as packaging of RNA, replication, movement and transmission, an antibody that disrupts epitopes involved in one of these processes may be successful in conferring resistance upon expression in plants. The principle of such "plantibody"-mediated resistance has been shown with a recombinant MAb against artichoke crinkled mottle virus by Tavladoraki and co-workers [31]. As the genes encoding the TSWV-specific scFv-antibodies were obtained using the phage display technique, they can be readily transferred into plants. Moreover, if later on mutants of TSWV evolve which potentially could overcome the antibody-mediated resistance, new inhibiting antibodies can be raised to ensure durable resistance, and in addition, to safeguard detection in plants. After all, the power of the phage display system is the selection of large panels of target-specific scFvs from naive combinatorial libraries, as was shown in this report in the case of TSWV.

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Chapter 6

Fluobodies: green fluorescent single-chain Fv fusion proteins

ABSTRACT

Expression of single-chain Fv antibodies and green fluorescent protein as fusion proteins (fluobodies) in *Escherichia coli* could facilitate the diagnosis of various diseases in single step assays based upon immunofluorescence and/or flow-cytometry. They might even replace some reagents frequently used in immunology such as chemically produced antibody-fluorochrome conjugates. Therefore, an expression system (pSKGFP) was designed that allows the expression of single-chain variable fragments as fusion proteins with modified green fluorescent proteins. The resulting green fluorescent single-chain Fv fusion proteins can be produced by expression in *E. coli* and purified in a single step via metal affinity chromatography. Two different single-chain variable fragment antibodies, both directed against the lipopolysaccharide of the bacterium *Ralstonia solanacearum* have been genetically fused to a red-shifted green fluorescent protein and the produced fusion protein was tested for usefulness in flow-cytometry and immunofluorescent cell staining. The fluobodies could be produced in cultures of bacterial cells and purified using immobilized metal affinity chromatography. They work well in flow-cytometry and immunofluorescent cell staining, are specific for their target antigens and unlike FITC-conjugated antibodies they do not fade upon illumination. The expression system is compatible to frequently used phage display vectors. Genes encoding single-chain antibody fragments can be easily transferred from these vectors into pSKGFP that allows single-step characterization of the selected recombinant antibodies by flow-cytometry or fluorescent cell staining.

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INTRODUCTION

Immunofluorescence (IF) [8,24] and phenotyping by flow-cytometry are useful techniques for localization studies or for the diagnosis of various diseases [18,23]. The fluorochrome fluorescein isothiocyanate (FITC) has been used extensively for this purpose. However, this fluorochrome is very sensitive to photobleaching by illumination. Moreover, conjugation to antibodies is not reproducible and if it occurs within the antigen binding site partial or complete loss of the antigen binding capacity might occur [23]. After all, lysine, the conjugation target for FITC is found frequently within antigen-binding sites [16]. Therefore, new alternative fluorochromes with comparable excitation and emission spectra but lacking the disadvantages of FITC have to be explored.

The green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, was recently described [3,14,22] and is a promising fluorescent probe with an emission peak at the same wavelength as FITC (510 nm). The wild-type GFP has two excitation peaks, a major one at 395 nm and a minor at 470 nm [32]. It was found to be very stable when excited at 480 nm [19,31]. Mutagenesis of wild-type GFP [4,5,6,10] led to improved versions of GFP. One of these mutants, GFPuv, had an increased expression level of GFP in bacteria and better solubility of the produced protein. Another mutant, GFPmut1 [4], had one single red-shifted excitation peak at 488 nm and contained two amino acid substitutions (Phe^{64} to Leu and Ser^{65} to Thr). It was found to fluoresce 35-fold more intensely than the wt-GFP when excited at 488 nm. Therefore, with the fluorochrome characteristics of FITC and an increased stability, this GFPmut1 spectral variant seems suited for application in IF and flow-cytometry.

Genetic fusion between antibody and GFP encoding genes would be ideal. It gives a 1:1 ratio between antibody and fluorochrome without the chance of inactivation. Recent progress in molecular immunology [9,13,21,26,30] has allowed cloning and expression of antibody encoding genes in *E. coli*. In this study, we describe the genetic fusion of the red-shifted (mut1) mutant of GFP to two recombinant, *Ralstonia solanacearum* (biovar 2, race 3) specific, single-chain variable fragment (scFv) antibodies [7,8] and the overexpression in *E. coli*. The use of these fluobodies was evaluated in IF and flow-cytometry.

MATERIAL AND METHODS

Mutating, cloning and production of a red shifted green fluorescent protein

To change the excitation peak of wild-type GFP or GFPuv (Clontech) from 396 nm to 488 nm [4], two amino acid changes were introduced by PCR (Fig. 6.1). Phe^{64} was replaced by Leu and Ser^{65} by Thr.

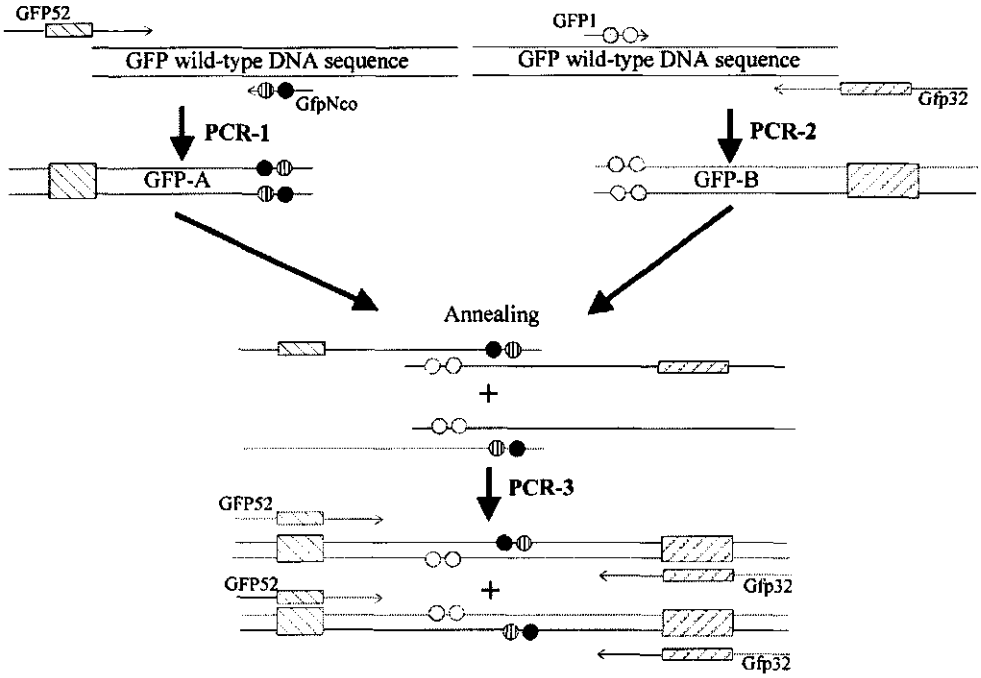

Figure 6.1. Simultaneous addition of restriction sites to, and side directed mutagenesis within the wild-type GFP DNA sequence by the polymerase chain reaction (PCR). Mutations (spheres) and restriction sites (boxes) are incorporated during 2 separate PCR steps, which yield the amplified DNA fragments GFP-A and GFP-B. As these fragments have overlapping DNA sequences, they can anneal and the complementary strands will be made by the DNA polymerase (PCR-3). However, within the transformed bacterium it is dependent on DNA mismatch repair whether or not a mutation is maintained. As the primers GFP1 and GFPNco were degenerate on the positions encoding for the cysteine residues, these mutations will only be present in a small fraction of the GFP transformants.

Briefly, from an *E. coli* culture, containing the GFP gene, 5 µl was taken and added to a 95 µl PCR mix containing 2.5 µM dNTPs; 0.25 U Super-Taq DNA polymerase (HT Biotechnology, Cambridge, UK); 2 mM $MgCl_2$ and 250 nM of either the primer combination GFP52 (5'-TTT TTA AGC TTC GCC ATG GCC GCG GCC GCA AGT AAA GGA GAA GAA CTT-3') and GfpNco (5'-TCT TGA AAA GCN CTG AAC ACC ATA AGT CAG AGT AGT GAC AAG TGT TGG CCA CGG AAC AGG-3') or primer combination GFP1 (5'-CTT ACC CTT AAA TTT ATT AGC ACT ACT G-3') and GFP32 (5'-GCT AGC GAA TTC CCT AGG TCA GTG ATG GTG ATG ATG GTG TTT GTA TAG TTC ATC CAT-3') in 50 mM Tris/HCl, pH 8. The PCR amplification (27 cycles: 1 min 94°C; 3 min 72°C, 1 min 40°C) was carried out in a thermal cycler (Perkin Elmer) and the PCR products, GFP-A (obtained with GFP52 x GFPNco) and GFP-B (obtained with GFP1 x GFP32) were gel-purified (Easyprep, Pharmacia, Uppsala, Sweden). From the purified fragments, GFP-A and

GFP-B, 2.5 µl was added to 95 µl PCR mix containing 2.5 µM dNTPs; 0.25 U Super-Taq DNA polymerase; 2 mM $MgCl_2$ in 50 mM Tris/HCl, pH 8. PCR cycling was carried out, without primers, for 10 cycles to allow the fragments to be joined by splicing by overlap extension [12].

To allow amplification of the mutated GFP genes, 20 additional cycles were carried out after addition of the outer primers GFP52 and GFP32. The obtained PCR products were digested after phenol extraction and ethanol precipitation, with *Hin*DIII and *Eco*RI. This was followed by ligation into *Hin*DIII/*Eco*RI digested pUC119 vector DNA. After transfection, the *E. coli* bacteria (XL1-Blue-MRF' Kan, Stratagene) were plated on selective LB-plates (100 µg Amp/ml) and grown overnight at 37°C.

Bacteria were screened for the presence of red-shifted GFPmut1 or GFPuv/mut1 mutants (Leu^{64} and Thr^{65}) by illumination with UV-light (395 nm) or light of 488 nm. Colonies which showed bright green fluorescence only when illuminated at 488 nm were picked, streaked and cultured in 100 ml of 2TY (containing 100 µg Amp/ml and 0.05 % glucose) at 37°C. When the OD_{600} reached 0.5, the temperature was decreased to 16°C. After 16 h, the bacteria were pelleted, the supernatant discarded and the bacteria were resuspended in 2TY medium (containing 100 µg AMP/ml) and grown for two additional days at 16°C.

The bacteria were pelleted and resuspended in 2 ml PBS + 0.1% Tween-20 to isolate the produced GFPmut1 or GFPuv/mut1 protein. The bacteria were lysed by repeated (5X) freezing and thawing [15]. The bacterial debris was removed by centrifugation and the supernatants were analysed for the presence of GFPmut1 protein by measuring the spectral properties with aid of a fluorometer. The produced (His)6-tagged GFP proteins were purified from the periplasmic fraction with immobilised metal affinity chromatography (IMAC) using Ni-NDA beads (Pharmacia, Uppsala, Sweden). Bound GFP or GFPuv/mut1 proteins were eluted with 0.1 M Tris/pH 8 containing 100 mM EDTA and stored at -20°C until use.

Production of scFv-GFP fusion proteins (fluobodies)

The improved GFP encoding sequences were digested out of the vector pUC119-GFPmut1 (Fig. 6.2A) with *Not*I and *Eco*RI, gel-purified and ligated into *Not*I/*Eco*RI digested pSKAP/S vector DNA [7], in frame with the scFvs anti-LPS12 [8] or anti-LPS7 [7]. The resulting vector pSK-GFPmut1 (Fig. 6.2B) was transfected to *E. coli* bacteria (XL1-Blue-MRF' Kan, Stratagene). Transformed bacteria were plated on selective LB-plates (100 µg Amp/ml) and grown overnight at 25°C. Individual colonies were tooth-picked and grown in 0.75 ml of LB-broth (100 µg Amp/ml) in 48 well plates (250 rpm). When the OD_{600} reached 0.5, the temperature was decreased to 16°C. After 1 h anhydrotetracycline was added to the medium (0.2 µg/ml final concentration) and growth was prolonged. After 48 h samples were taken and the fluorescence at 488 nm was measured with a fluorometer (Perkin Elmer 7700).

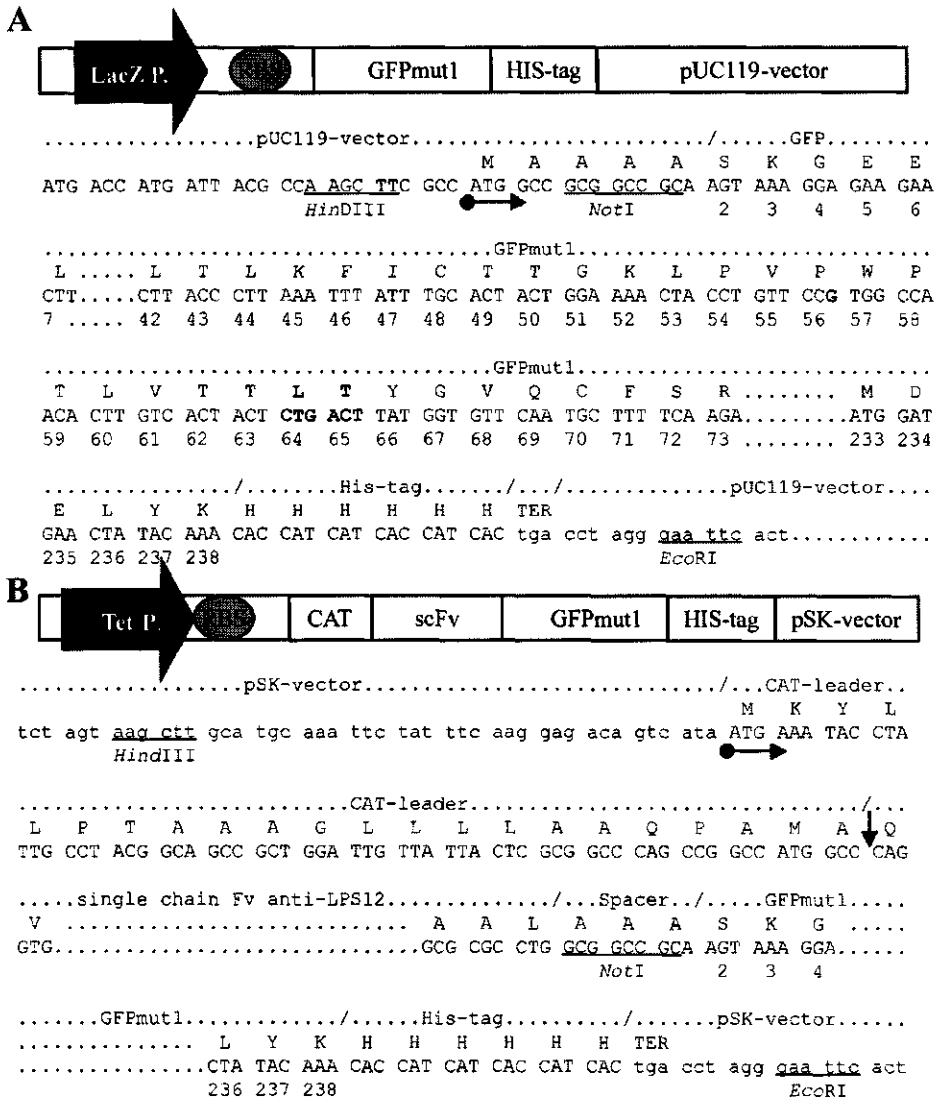

Figure 6.2. A) Schematic representation of the vector pUC119-GFPmut1. The positions of the *Lac*Z promoter, the ribosomal binding site (RBS), the GFPmut1 sequence and the His(6)-tag are indicated. Within the nucleotide sequence, the important restriction sites are underlined and the nucleotides that differ from the wild-type sequence are in bold face. The start codon of translation is indicated by the horizontal arrow and the termination-codon by TER. Numbering of amino acids is according to the wild-type GFP protein sequence. **B)** Schematic representation of the vector pSK-GFPmut1. The position of the *Tet* promoter (inducible with anhydrotetracycline), the RBS, the chloramphenicol acetyltransferase leader sequence (CAT), the single-chain Fv (scFv), the GFPmut1 sequence and the His(6)-tag are indicated. The important restriction sites are underlined, and within the nucleotide sequence the nucleotides that differ from the wild-type sequence are in bold face. The start-codon for translation is indicated by the horizontal arrow and the termination codon by TER. Numbering of amino acids is according to the wild-type GFP protein sequence. The site were the CAT-signal peptide is cleaved from the scFv-GFP fusion protein is indicated by the vertical arrow.

Purification of fluobodies

Cultures were grown, induced and pelleted as described above. After incubation (5 min, 0°C) of the bacteria with 1/20 volume (referring to the original culture size) of a 50 mM Tris/HCl pH 8 buffer containing, 30% sucrose and 1 mM EDTA, the produced proteins were extracted (45 min, 0°C) from the periplasm with 1/20 volume of 5 mM $MgSO_4$. The produced (His)6-tagged proteins were purified from the periplasmic fraction with immobilized metal affinity chromatography (IMAC) using Ni-NDA beads (Pharmacia, Uppsala, Sweden). Bound proteins were eluted with 0.1 M Tris/pH 8 containing 100 mM EDTA and stored at 4°C.

SDS-PAGE and Western blotting

Purified GFP and fluobodies were loaded on a 12.5 % SDS polyacryl amide gel and blotted onto nitro-cellulose according to Towbin and co-workers [27]. After blocking for 30 min with PBS (containing 5% skimmed milk powder and 0.1 % Tween-20) the Western blot was incubated (1.5 h, room temperature) with GFP specific polyclonal rabbit antibodies (Clontech) diluted 1:25,000 in PBS (containing 2% skimmed milk powder and 0.1 % Tween-20). After washing (4 x, PBS 0.1 % Tween-20) the blot was incubated for 1 h with alkaline phosphatase conjugated Goat anti-Rabbit polyclonal antibodies (diluted 1:5,000 in PBS containing 2% skimmed milk powder and 0.1 % Tween-20). Finally, staining with 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium substrate (BCIP/NBT) was used to visualize the bands on the Western blot.

Flow-cytometry

Purified GFPmut1 or scFv LPS12-GFPmut1 fluobodies were added to suspensions of *Ralstonia solanacearum* or *Ralstonia picketii* (10^7 bacteria/ml PBS, containing 0.1% Tween-20 and 5% FCS) and incubated for 1 h while rotating. Bacteria were washed twice with PBS + 0.1 Tween-20, resuspended in 1.5 ml PBS and analyzed for fluorescent staining. From each sample 10,000 bacteria were screened, using a fluorescent activated cell sorter.

Immunofluorescence

IF was performed according to Van der Wolf and co-workers [29]. For IF cell-staining the bacteria were coated on microscope slides and incubated with GFPmut1, scFv LPS7-GFPmut1 fluobodies or with a *R. solanacearum*-specific FITC-conjugated polyclonal rabbit antiserum.

DNA Sequencing

The nucleotide sequences of the GFP inserts in pUC119 were verified by the dideoxy chain termination method [25] on an A.L.F. DNA sequencer (Pharmacia).

RESULTS

The *Aequorea* wt-GFP gene was modified by PCR to generate the GFPmut1 spectral mutant with a single excitation peak at 488 nm. In addition, a his-tag was added to the C-terminus of the GFP protein and convenient restriction sites for cloning into the pUC119 vector were added. Moreover, an attempt was carried out to change the cysteine residues on positions 48 and 70 by PCR, as it was anticipated these that would interfere with secretion of functional scFv-GFP fusion proteins into the periplasmic space.

Several bacteria obtained from transfection of the pUC119-GFPmut1 vector DNA (Fig. 6.2A) showed fluorescence only when illuminated at 488 nm (i.e. production of red-shifted GFP). The GFPmut1 proteins could be separated from the extracted cytoplasmic fraction by immobilized metal chromatography and yielded up to 2 mg of GFPmut1 protein per liter of bacterial culture. The GFPmut1 encoding plasmids were isolated from the most intensely fluorescing colonies and the two amino acid changes in the GFPmut1 region, Phe[64] to Leu and Ser[65] to Thr, were confirmed by DNA-sequencing. The cysteine residues on positions 48 and 70 were conserved, despite the attempt to replace them.

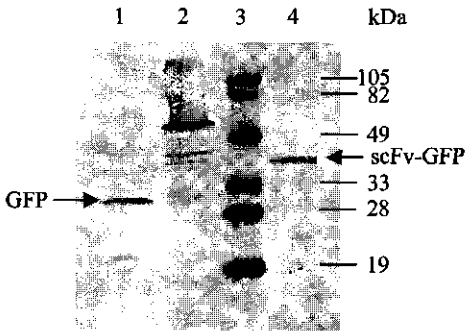

Figure 6.3. Western blot on GFP containing samples. Proteins in total lysates of GFPmut1 (lane 1) or scFv-GFPmut1 (lane 2) producing bacteria, molecular weight markers (lane 3) or purified scFv-GFPmut1 fraction (lane 4) were separated by SDS-PAGE, blotted to nitrocellulose and stained with GFP-specific antibodies.

The GFPmut1 encoding genes were digested from the GFPmut1 plasmids (Fig. 6.2A) and genetically fused to a gene encoding the scFv anti-LPS12 [8] (Fig. 6.2B) which is highly specific for the lipopolysaccharide of the bacterium *R. solanacearum* (biovar2, race 3). Upon induction with anhydrotetracycline clear green fluorescence could be measured intracellularly bacteria with the aid of a fluorometer. Further analysis showed that a green fluorescent compound was present in the periplasmic fraction. Purification of scFv-GFPmut1 fusion proteins from the periplasmic fraction by immobilized metal affinity chromatography and subsequent analysis by Western blotting with GFP-specific antibodies showed an intensive band (Fig. 6.3, lane 4). With a molecular mass of approximately 45 kDa it was larger than the 30 kDa observed for non-fused GFPmut1 protein (Fig. 6.3, lane 1) but smaller than the 53 kDa which was calculated using the scFv LPS12-GFP protein sequence. This phenomenon was probably the result of the formation of a functional chromatophore within the scFv-GFP

fusion protein and has been observed previously [2]. Support for this assumption was obtained as on Western blot, within a total lysate of the scFv-GFP producing bacteria, a major band of 55 kDa was observed together with two additional bands of 47 and 45 kDa. The observed molecular masses correspond to 3 products of the scFv-GFPmut1 fusion protein: scFv-GFPmut1, preceded by the chloramphenicol acetyl transferase (CAT)-signal peptide (55 kDa); scFv-GFPmut1, preceded by the CAT-signal peptide with a functional chromatophore (47 kDa) and the desired periplasmic secretion product scFv-GFPmut1, with an active fluorochrome but without the CAT-signal peptide (45 kDa).

The quantity of the produced scFv-GFPmut1 proteins was on average 100 µg per liter of bacterial culture. Replacement of the GFPmut1 encoding sequence by GFPuv/mut1 did not result in the expected improvement of expression and enhanced secretion of the scFv-GFP fusion protein to the periplasm, but rather lowered the yield.

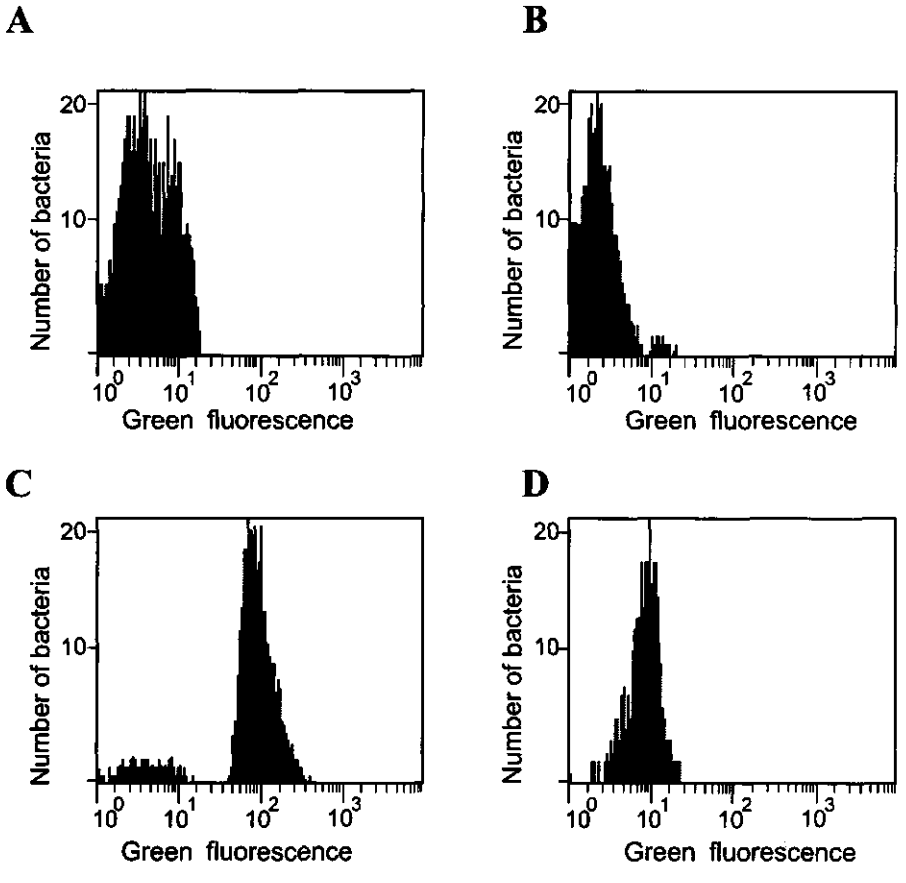

Figure 6.4. Flow-cytometry analysis of the binding of GFPmut1 to **A)** *Ralstonia solanacearum* bacteria and **B)** *R. picketii* bacteria and of the binding of fluobody scFv LPS12-GFPmut1 to **C)** *R. solanacearum* and **D)** *R. picketii.*

The bifunctional character of the scFv-GFPmut1 fusion proteins was shown by flow-cytometry (Fig. 6.4). Comparable amounts of either non-fused GFPmut1 protein or anti-LPS12 fluobodies were applied to *R. solanacearum* bacteria. Binding, corresponding to a gain in immunofluorescence, was measured by flow-cytometry. The fluobodies showed, in contrast to the GFPmut1 protein (Fig. 6.4A), bright fluorescent staining of the target bacteria (Fig. 6.4C). This staining was specific as the negative control, *R. picketii* bacteria (closely related to *R. solanacearum*), was not stained with GFPmut1 proteins (Fig. 6.4B) or with anti-LPS 12 fluobodies (Fig. 6.4D).

The applicability of the GFPmut1-scFv fusion proteins in IF was shown. Bright staining, comparable to a *R. solanacearum* specific polyclonal antiserum, of *R. solanacearum* bacteria was observed upon binding with anti-LPS 7 [7] fluobodies (Fig. 6.5). This reaction was specific because *R. picketii* bacteria were not stained by anti-LPS 7 fluobodies (data not shown). Moreover, in IF the advantage of fluobodies over FITC-conjugates was shown. While bacteria labeled with antibody-FITC conjugates were bleached completely after 4 min of illumination (Fig. 6.5B), bacteria labeled with fluobodies remained visible with a fluorescent microscope without any sign of bleaching (Fig. 6.5A).

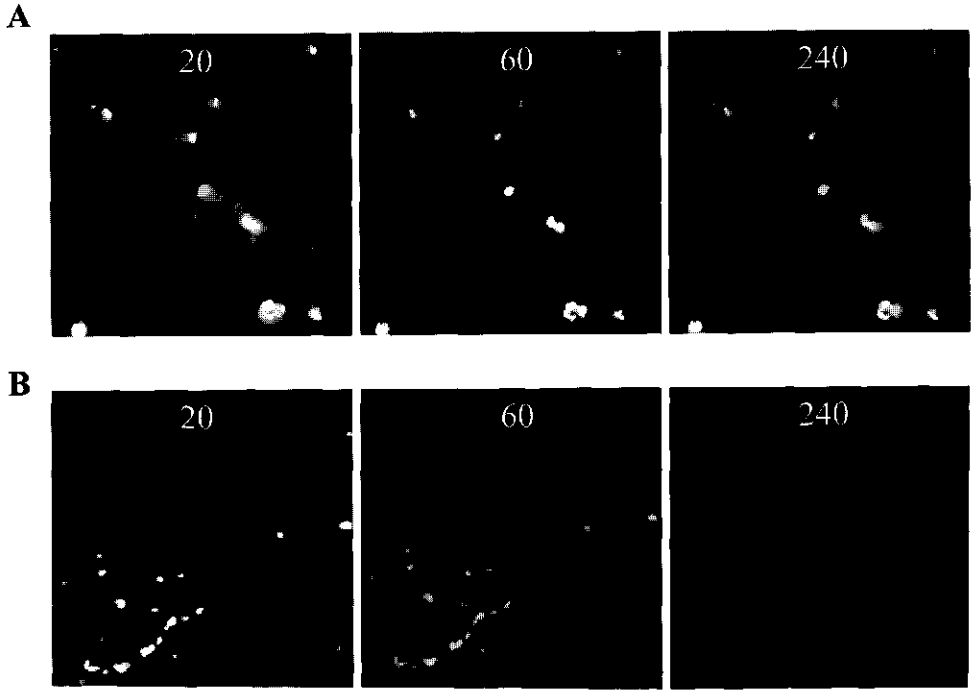

Figure 6.5. Immunofluorescence cell staining of *Ralstonia solanacearum* bacteria using **A)** fluobody scFv LPS7-GFPmut1 or **B)** FITC conjugated *R. solanacearum* specific polyclonal antiserum. Photographs were made after illumination of the objects for 20, 60 or 240 seconds, using the same photographic exposure time.

DISCUSSION

Fluorescent antibodies, which can be applied for direct labeling in flow-cytometry or in immunofluorescence experiments, can be obtained from *in vivo* expression in *E. coli* bacteria. The restriction sites in the vector allow easy transfer of scFv-genes from phage display vectors like pHen1 [11] and pCantab series (Pharmacia).

The general yield of obtained fluobodies is not high. While for non-fused scFvs the maximum yield score of 3 mg per liter bacterial culture can be reached in shaker flasks under non-optimal conditions [8], the genetic fusion with GFPmut1 drastically reduced the yield. This while under the same conditions the non-fused GFPmut1 protein, when produced in the cytoplasm (i.e. without secretion to the periplasm), reached a yield of 2 mg per liter. In addition, when the same GFPmut1 sequence was preceded by a signal sequence like pelB [17], the fluorescence dropped to a similar level as if a scFv was present. The use of another red-shifted GFP mutant (GFPuv/mut1) sequence, optimized for expression in *E. coli*, increased the yield when expressed as a non-fused protein in the cytoplasm. But when preceded by either a signal peptide alone or a signal peptide in combination with a scFv the yield also dropped, even to levels below those obtained with the GFPmut1 protein. Apparently, the fast folding characteristics of the GFPuv/mut1 mutant further lowers the ability of the protein to be secreted.

It was anticipated that problems could arise after secretion of the scFv-GFP fusion proteins into the periplasmic space, as 2 cysteine residues are present in the wt-GFP protein. These cysteine residues normally do not form disulfide bonds in the wt-GFP but they might cause cross-links [28] with the cysteine residues of the scFv-domain. Therefore, the primer sequences were designed to replace those cysteine residues by serine, glycine or arginine. However, in all cases, DNA-sequencing revealed that both cysteine residues were conserved. As no non-fluorescing clones were sequenced it was not proved that both cysteine residues are required for the formation of a functional fluorochrome. It appeared that no internal disulfide bonds were formed between the scFv- and GFP-domains as the produced protein was able to bind to the target antigen and simultaneously to function as a highly stable fluorescent dye.

High expression of scFv-GFP fusion proteins in the cytoplasm of *E. coli* might be possible as high yields were described for a genetic fusion of GFP with protein A [2]. This fusion protein was extracted by total disruption of the bacteria through sonic oscillation. For scFv-GFP fusion proteins this is not favorable as the required disulfide bonds are not formed in the cytoplasm and thus extracted scFv would be unstable. However, secretion of GFP fusion proteins is possible, as was demonstrated by Oker-Blom and co-workers [20] for the genetic fusion of GFP with streptavidin. This GFP-streptavidin fusion protein was expressed at high yields in insect cells and appeared to be secreted into granules. Therefore, expression of fluobodies might give a better yield in insect cells or in other eukaryotic expression systems in which the folding and secretion of proteins is guided by more adequate chaperone proteins.

Although the GFP-streptavidin and GFP-Protein A fusion proteins have expression advantages over the scFv-GFP fusion protein, they require multiple incubation steps in immunoassays whereas scFv-GFP fusion proteins can be used in a single-step assay. Together, the data show that fluobodies are a promising tool as fluorescent reagents in immunoassays. Especially, since exploitation of fluobodies with other spectral characteristics might facilitate multiple labeling [1,33] of different epitopes simultaneously.

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Chapter 7

Discussion, Summary and Concluding remarks

This thesis describes the applicability of the novel phage display technique to select plant-pathogen-specific monoclonal antibodies (MAbs) from combinatorial antibody libraries. The retrieved MAbs are so specific that they can be used as diagnostic tools in sensitive immunoassays for the detection and identification of plant pathogens. Testing results, obtained from laboratories that have applied these recombinant MAbs, are discussed in this conclusive chapter.

Background

In the last decades, it has become clear that chemical crop protection has to be reduced. Many of the pesticides, applied to destroy plant-pathogenic fungi, bacteria, insects and nematodes, are hazardous to the environment, and form a serious health risk for animals and humans. Since breeding for disease resistance generally takes years, epidemics have to be prevented by sanitary measures in combination with diagnosis in an early stage of disease development or preferably beforehand. Consequently, sensitive diagnostic assays are required that allow healthy plant propagation material to be certified and soil to be monitored. It is obvious that these data can be used to take adequate crop management decisions.

The problems associated with serological detection of plant pathogens

Immunoassays are widely applied as detection methods in agriculture because they can be applied to large numbers of samples, and their application is fast, robust, sensitive and cheap. A major concern is the specificity of the assays. Polyclonal antisera are still the active ingredient of many useful immunoassays. However, the immunochemical complexity of several target organisms is the main reason that extensive cross-reactions with non-target organisms can occur when these antisera are used.

In 1975, Köhler and Milstein showed that this problem could be circumvented by making monospecific antibodies in continuous cultures by fusing the antibody producing B-lymphocytes with myeloma cells [8]. This 'hybridoma' technique opened a new perspective for the production of specific monoclonal antibodies (MAbs) against various antigens. Today, many MAbs are used in research, as therapeutic agent and for diagnostic purposes.

However, the hybridoma technique was not as advantageous as was expected in the field of plant-pathogen detection. From the trials in raising specific MAbs against many different plant-pathogens, it became apparent that there are 'difficult' antigens. Several plant viruses can not be sufficiently purified and contain immunodominant plant residues and, as a consequence, the immune response is mainly directed to those impurities. In addition, plant-pathogenic bacteria, fungi and nematodes contain many epitopes that are 'shared' with closely related non-pathogenic family members, which is often leading to extensive cross-reactions. Therefore, the selection of the cells producing the desired MAbs is laborious and often impossible.

Many techniques have been described to deal with 'difficult' antigens and varied from the enrichment of the desired B-cell populations to guiding of the immune response towards the target antigen, such as:

- Immunoadsorption of antigen-specific B-cells [2].
- Selection of antigen-specific B-cells by fluorescence activated cell sorting [15].
- Complement-mediated lysis of undesired B-cells [2].
- Masking (immuno-complexing) of contaminating immunodominant plant epitopes with anti-healthy plant antibodies during immunization [2].
- Suppression of the immune response to healthy plant extracts by injection with cyclophosphamide or other immuno suppressive drugs prior to immunization [11,16].
- Induction of immunological tolerance through tolerisation of neonatal mice with healthy plant extracts prior to immunization [6].

Although these methods have occasionally shown their value in obtaining specific MAbs in their respective cases, the common drawback of all these methods is that they are not generally applicable. In addition, the latter two are also repulsive from an ethical point of view.

Bypassing immunization: A general solution to deal with 'difficult' antigens

A way to circumvent immunization was offered by recent developments in recombinant DNA techniques [14,17]. These techniques allow cloning and expression of large naive antibody repertoires, derived from non-immunized human donors, in *E. coli* as single-chain antibodies (scFvs) or Fab fragments. The use of these combinatorial antibody libraries in combination with the display of functional antibody fragments at the tips of filamentous phage created a powerful selection system for MAbs [5,12]. Utilization of this phage display system allows direct selection of highly specific MAbs from naive combinatorial antibody libraries [3,13,18]. As immunization is omitted, phage display is not biased. Hence it will not lead to antibodies directed against the most immunodominant epitope but rather towards the most abundant antigenic determinant.

Mission impossible: how to make the impossible possible

Because application of the hybridoma technique was not successful in generating MAbs of sufficient specificity to detect the plant-pathogen *Ralstonia solanacearum* in reliable and sensitive diagnostic assays, the efficacy of the phage display system was challenged (Chapter 2). To achieve this, phages derived from the (naive) Vaughan combinatorial antibody library (1.4×10^{10} different scFvs) were panned against purified lipopolysaccharides (LPS) which were isolated from *R. solanacearum* race 3 bacteria. After four successive rounds of phage growth and selection for LPS binding, soluble scFvs were produced and tested by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy (IF). Four different scFvs could be distinguished on bases of RFLP analysis. Characterization of the monoclonal scFvs against several bacterial strains, indicated a specificity for *R. solanacearum* (biovar 2, race 3) LPS which is higher than can be obtained with conventional polyclonal antisera. The fact that the four scFvs were obtained within six weeks after starting this study emphasizes the potency of the phage display system.

Selection of specific antibodies from the synthetic Nissim library, containing over 10^8 different scFv, against beet necrotic yellow vein virus (BNYVV) was achieved (Chapter 3) through expression of the antibody fragments on the surface of bacteriophage M13 and subsequent binding of this phage-antibody to immobilized BNYVV. After several rounds of selection seven BNYVV-specific recombinant monoclonal antibodies were obtained. However, the yield of these monovalent scFv antibodies was low. In an attempt to improve the yields, the genes encoding the BNYVV-specific scFvs were genetically fused to alkaline phosphatase (AP/S) and expressed in *E. coli*. However, out of the seven different anti-BNYVV scFv-AP/S fusion proteins only three showed alkaline phosphatase activity and retained affinity for BNYVV and the quantity of produced scFv-AP fusion proteins was found to be low.

Analysis of scFv encoding DNA during expression in *E. coli* showed the occurrence of high plasmid loss and a high incidence of recombination within the scFv encoding DNA, both for scFv and scFv-AP/S fusion proteins. Probably the scFvs are toxic and offer bacteria that recombine the plasmid DNA (and thereby disable expression of scFvs) a growth advantage. The more tightly repressed tetracycline promoter was used to replace the "leaky" LacZ promoter (Chapter 4). The co-expressed repressor protein tightly repressed the tetracycline promoter in the absence of inducer (anhydrotetracycline). When this new expression system was compared to the old expression system (LacZ induction by IPTG) for expression of scFv-AP fusion proteins, it was found to be superior as improved yields of functional recombinant antibodies were obtained. The tetracycline promoter is also more convenient to use. This is evident when large cultures or high numbers of cultures are grown because the medium has not to be changed since the use of this promoter is, unlike the LacZ promoter, glucose independent.

Selection of tomato spotted wilt virus (TSWV)-specific scFvs from the naive Vaughan combinatorial antibody library (Vaughan) against purified nucleocapsids and against purified complete virus was also successful (Chapter 5). In contrast to previous selections the pooled scFv encoding DNA was isolated after the fourth round of selection, inserted in the vector pSKAP/S (Chapter 4) and tested directly as individual scFv-AP/S fusion proteins. Twelve different scFvs were obtained against the nucleocapsid (N) and five against the glycoproteins, G1 or G2. Six of the derived antibodies were produced with good yields: four scFvs against the nucleoprotein and two scFvs against G1 or G2. In ELISA they reacted with TSWV proteins in infected *Nicotiana benthamiana* and not with healthy plant extracts. When the N-reactive scFvs were evaluated for their specificity against six other tospoviruses, cross-reactions were observed with tomato chlorotic spot virus and to a lesser extent with groundnut ringspot virus. Several of the TSWV-reactive scFvs might be useful in routine testing, and an ELISA based on these recombinant antibodies is presently evaluated by routine testing laboratories.

The ultimate goal

The development of a sensitive double antibody sandwich ELISA format, based on recombinant antibodies, was a major objective. Whole antibodies coat very well to ELISA plates because they contain flexible hinge-regions that allow rotation of the antigen-binding site. However, scFvs are not suited for coating onto ELISA plates, as they are small molecules that are inactivated upon coating [7]. Fusion of scFv to immunoglobulin domains may enhance the coating efficiency while retaining the full binding activity. Therefore, N56, one of the TSWV binding scFvs, was transformed into a mini-antibody through addition of a constant part (Mouse K constant region) and a flexible dimerization domain (Chapter 5). These bivalent mini-antibodies proved to be very active as a coating reagent in ELISA (Chapter 5) and could compete with a conventional polyclonal antiserum for coating efficiency. As was shown in Chapter 5, sensitive detection of TSWV nucleocapsids could be

achieved by application of scFv-AP/S fusion proteins as antibody-enzyme conjugate. Therefore, serological testing can thus be carried out entirely by using bacteria derived coating and detection reagents.

Endowing antibodies with novel properties

Routine testing for *R. solanacearum* is currently mainly performed by immunofluorescent cell staining (IF). However, conjugation of antibodies with the fluorochrome fluorescein isothiocyanate (FITC) is not reproducible and FITC fades rapidly upon illumination. This is not the case with the product of he genetic fusion of *R. solanacearum*-specific scFvs with green fluorescent protein (GFP). Bright, specific fluorescent labeling of target bacteria was observed when the obtained scFv-GFP fusion proteins (fluobodies) were tested by flow cytometry and in IF. The fluobodies proved to be more resistant to illumination, as was shown by IF after prolonged illumination (Chapter 6).

Table 7.1. Summary of testing the recombinant scFv alkaline phosphatase fusion protein (monoclonal) in comparison to polyclonal anti-*Ralstonia solanacearum* antiserum FITC or alkaline phosphatase conjugates. For ELISA the plates were coated with a *R. solanacearum* specific antiserum (pca 9523bcd), blocked, incubated with samples (obtained from potato lots by PD regulations), incubated with either LPS7-AP/S or pca9523bcd-AP conjugate.

potato samples N=206	IF-polyclonal			ELISA-polyclonal		ELISA-monoclonal	
	IF+ Typical	IF+ Atypical	IF-	60 min OD>350	60 min OD<350	60 min OD>150	60 min OD<150
[1]NAK97 IF+, PD97+, Undiluted, N=19	100 %	0 %	0 %	100 %	0 %	100 %	0 %
NAK97 IF+ ,PD97+, Diluted 1:10, N=17	100 %	0 %	0 %	94 %	6 %	100 %	0 %
[2]Suspected, NAK97 IF±, PD97-,Undiluted, N=31	16 %	13 %	71 %	32 %	68 %	26 %	74 %
Suspected NAK97 IF±, PD97-, Diluted 1:10, N=27	15 %	11 %	74 %	19 %	81 %	15 %	85 %
[3]NAK97 Cross-reactions N=52	0 %	12 %	88 %	4 %	96 %	0 %	100 %
Negative N=47	0 %	2 %	98 %	0 %	0 %	0 %	100 %
PD negative control N=13	0 %	0 %	100 %	0 %	0 %	0 %	100 %

[1]Samples were tested positive in 1997 and it was confirmed that they contained *R. solanacearum* bacteria.
[2]Samples were tested positive in 1997 but did not contain *R. solanacearum* bacteria.
[3]Samples were tested negative in 1997.

Preliminary data of routine testing by diagnostic laboratories

The *R. solanacearum*-specific scFv, anti-LPS 7, was evaluated for its use in IF and in ELISA and was found to react with the same specificity as scFv LPS 12 (Chapter 2). It reacted with several *R. solanacearum* race 3 strains and showed cross-reactions with fewer strains than the polyclonal antiserum that is routinely applied for brown rot diagnosis of potato in the Netherlands. The general Netherlands inspection service for potatoes (NAK) evaluated the use of the anti-LPS7-AP/S based ELISA in their own laboratory and compared the results

with those obtained with IF. The samples, obtained and already tested by the Dutch Plant Protection Service (PD) and NAK in 1997, were derived from various potato varieties such as Agria, Bildstar, Bintje Cardinal, Desiree, Diamant, Dore, Elkana, Felsina, van Gogh, Kanjer, Karnico, L. Rossetta, Monalisa, Spunta, Stefano and Symphony. The extraction of bacteria was performed according to the regulation of the PD and the results, summarized in Table 7.1, show that IF and ELISA gave comparable results for detection. Moreover, the ELISA that utilizes scFv-AP/S fusion proteins for detection is comparable with the polyclonal ELISA, but more importantly, the background is lower when the recombinant antibody is used for detection instead of the polyclonal alkaline phosphatase conjugate. No problems of increased background were observed regarding the sampling from the different potato varieties. The reduced number of cross-reactions that was observed for the recombinant antibodies in Chapter 2 also seems to hold for this field trial, as the number of positive samples found within the pool of suspected and cross-reacting samples was reduced. It can therefore be concluded that the recombinant scFv LPS7 is very useful under real testing conditions.

General conclusion and future perspectives

This thesis shows that antibody phage display is a useful technique for the selection of specific recombinant antibodies against a variety of plant-pathogens. Thus far, recombinant antibodies have been selected only against viruses and bacteria. The results described are promising, and it is expected that the phage display technique can also be used successfully for raising specific MAbs against fungi and nematodes. Therefore, the perspective that was predicted after introduction of the hybridoma technique in 1975 [8], now becomes reality with application of the novel antibody phage display technique.

Although the synthetic naive Nissim library (10^8) was not compared directly to the naive Vaughan library (10^{10}), the obtained results favor the application of the latter. The variability within the retrieved scFv-antibodies was higher and the yield of the scFvs was better. Moreover, the amount of scFv-AP/S fusion protein required for a strong signal in the ELISA was up to 50-fold lower, indicating that the recombinant antibodies retrieved from the Vaughan library were of higher affinity. In general, it can be stated that large ($>10^{10}$) naive antibody libraries are a prerequisite for generating useful recombinant antibodies.

More than 50 different antigen-specific recombinant antibodies were selected without the use of experimental animals. However, screening the selected scFv-antibodies was a major problem since *E. coli* produced several of those, only with great difficulty. It remains disappointing that the choice of a scFv for further characterization was based more on its yield, than on its affinity and specificity for the antigen. Development of eukaryotic expression systems might be an option. Yeast, insect-cells, fungi and plants resemble more closely the mammalian cells than the prokaryotic bacterium *E. coli* with regard to protein expression, and improved yields of functional scFv-antibodies can be expected when eukaryotes are used.

There is an ongoing improvement of selection techniques from combinatorial antibody phage display libraries such as selectively infective phage [1,9], expression of antibodies on ribosomes [4] and selectively infected bacteria [10]. Meanwhile, combinatorial phage display libraries are designed to give better folding, and consequently, better producing antibodies. When these novel techniques find their way into plant pathology, it will undoubtedly result in highly specific antibodies that can be used in diagnostic assays that are fast, reliable, robust and cheap.

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Samenvatting

Achtergrond

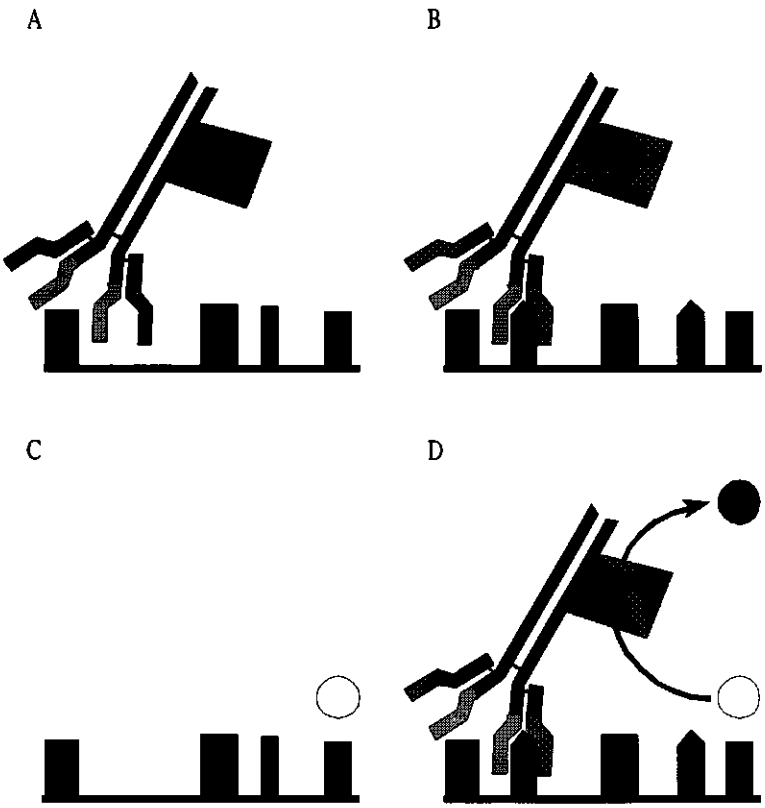
In de laatste decennia is het steeds duidelijker geworden dat het gebruik van chemische bestrijdingsmiddelen sterk verminderd moet worden. Veel van deze pesticiden zijn zeer belastend voor het milieu en vormen een gezondheidsrisico voor mens en dier. Het is dus zaak om milieuvriendelijke methoden te ontwikkelen om de plantenziekten in de hand te houden. In dit verband is het van belang dat het uitgangsmateriaal (knollen, bollen, zaad en entmateriaal) vrij is van plantenpathogenen. Het optreden van ziektes zal dan in de regel achterwege blijven en de bestrijding van ziektes is niet of veel minder nodig. Voorts kan door het toetsen van grondmonsters informatie verkregen worden die te gebruiken is voor advies met betrekking tot gewasrotatie, cultivar selectie, toepassing van pesticiden, moment van oogsten en nabehandeling van het geoogste gewas.

Toetsmethoden

Voor het opsporen van ziekteverwekkers (plantenpathogenen) zoals bijvoorbeeld virussen, bacteriën, schimmels, insecten of parasitaire nematoden in zaaigoed, planten of grond, zijn betrouwbare toetsingsmethoden nodig. Er moet immers met zekerheid vastgesteld kunnen worden of plant- of grondmonsters besmet zijn of niet. Een methode die vaak wordt gebruikt, werkt met behulp van antilichamen uit het serum van dieren die opzettelijk zijn ingespoten met het betreffende plantenpathogeen. De dieren worden hier zelf niet ziek van, maar produceren wel grote hoeveelheden zogenaamde antilichamen. Deze antilichamen kunnen vervolgens in een serologische toets worden gebruikt (Fig. 1). Dergelijke serologische toetsen zijn niet alleen toepasbaar op grote aantallen monsters, ze zijn bovendien snel, robuust en goedkoop.

Specificiteit van ons afweersysteem

Bepaalde bacteriën, virussen en schimmels zijn in staat om mens en dier ziek te maken. Meestal wordt de patient na korte tijd weer beter. Dit komt omdat bepaalde cellen (witte bloedlichaampjes) van het afweersysteem op de ziekteverwekker reageren door onder andere antilichamen te maken tegen een scala aan structuren (epitopen) van deze lichaamsvreemde stoffen. Deze polyklonale antilichamen worden vervolgens afgegeven aan het bloed en kunnen de ziekteverwekker elders in het lichaam herkennen en deze vervolgens vernietigen. De lichaamscellen, die de antilichamen produceren, hebben een geheugenfunctie. Als je eenmaal de mazelen gehad hebt, krijg je gedurende de rest van je leven nooit meer mazelen. Dat andere ziekteverwekkers nog steeds kunnen toeslaan komt doordat de antilichamen, die gemaakt zijn tegen het mazelenvirus, bijvoorbeeld het griepvirus niet herkennen. Een antistof heeft dus een bepaalde specificiteit. Omdat ziekteverwekkers in talloze soorten en variaties in de natuur voorkomen beschikt het afweersysteem dan ook over een groot aantal verschillende antilichamen, elk met een andere specificiteit. Er wordt geschat dat het afweersysteem meer dan 1 miljard antilichamen kan maken, elk met een verschillende specificiteit!



Figuur 1. Een schematische weergave van een serologische toets. Extracten van gezonde planten (**A**) en zieke planten (**B**) worden gekoppeld aan het oppervlak van een vaste drager. Vervolgens worden antilichamen tegen de ziekteverwekker toegevoegd. Als de ziekteverwekker aanwezig is zal de antistof aan de ziekteverwekker binden (**B**). Deze binding is zo sterk dat na grondig wassen alle antilichamen die bij de gezonde plant werden toegevoegd (**A**) worden weggewassen (**C**) maar niet bij de zieke plant (**D**). Na het wassen wordt een chemische stof toegevoegd. Deze stof is kleurloos (○)maar wordt door het enzym alkalische fosfatase (AP) dat gekoppeld is aan de antistof omgezet in een kleurstof (●). De zieke planten zijn dus detecteerbaar door een kleuromslag in de test.

Helaas zijn de polyklonale antilichamen die in het bloed aanwezig zijn vaak niet specifiek genoeg om toegepast te kunnen worden in een detectiesysteem. Ze zijn niet gericht tegen één bepaalde epitoop van de ziekteverwekker maar tegen een breed scala daarvan. Enkele van die structuren kunnen ook voorkomen bij andere, vaak onschadelijke, organismen die dikwijls aanwezig zijn in de directe omgeving van de plant. Het is dus zaak om alleen die antilichamen te produceren, die onderscheid kunnen maken tussen zieke en gezonde planten.

Productie van antilichamen in de reageerbuis.

Technisch is het mogelijk om de geactiveerde antistof-producerende cellen van het afweersysteem te isoleren en ze te kloneren doormiddel van een celfusie met kankercellen. Hoewel er bij een dergelijke celfusie vele duizenden antistof-producerende cel-klonen

ontstaan, is dit aantal in veel gevallen toch niet toereikend om hieruit een kloon te selecteren die antilichamen met de vereiste specificiteit produceert. In de praktijk blijkt namelijk vaak dat het selecteren van een kloon die de gezochte specifieke antistof produceert vergelijkbaar is met het zoeken van een naald in een hooiberg. De inefficiënte celfusie en de eigenschap van het afweersysteem, om juist tegen sommige epitopen sterk te reageren en tegen andere niet, liggen hieraan ten grondslag.

Recombinant-DNA techniek: de uitkomst!

Een uitkomst voor deze problemen werd geboden door de toepassing van moderne recombinant-DNA technieken. Deze technieken maakten het mogelijk om het genetische materiaal, dat de blauwdruk vormt voor de antilichamen, over te brengen van de antistof producerende cellen naar bacteriën. Dit resulteerde uiteindelijk in zogenaamde antilichaam-bibliotheken die de blauwdrukken bevatten voor meer dan 10 miljard verschillende antilichamen. Deze blauwdrukken kunnen hier naar believen uitgehaald worden door de bacteriën te infecteren met een bacterie-virus (bacteriofaag). Tijdens de vermeerdering van het virus in de bacterie neemt deze niet alleen één blauwdruk voor één antistof in zich op maar met behulp van de bacterie wordt deze antistof ook geproduceerd en vervolgens aan het oppervlak van het virus ingebouwd. Na een strenge selectie uit deze miljarden verschillende bacteriofagen op specificiteit kan dit uiteindelijk een monoklonaal antilichaam opleveren welke gebruikt kan worden om de gezonde van de zieke plant te onderscheiden. Een bijkomend voordeel is dat voor de selectie van deze monoklonale antilichamen géén proefdieren meer nodig zijn.

Toetsen op plantenpathogenen

Het hoofdonderwerp van dit proefschrift is de productie en selectie van plantenpathogeen specifieke monoklonale antilichamen, die bruikbaar zijn in serologische toetsen. Daarbij werden met behulp van recombinant-DNA technologie monoklonale antilichamen geselecteerd die specifiek gericht zijn een tegen plantenpathogeen zoals de bacterie *Ralstonia solanacearum* (biovar 2, race 3), de veroorzaker van bruinrot bij de aardappel (Hoofdstuk 2) en het "beet necrotic yellow vein virus" (BNYVV), de veroorzaker van rhizomanie bij de suikerbiet (Hoofdstuk 3). Verder is er aandacht besteed aan de optimalisatie van productie en toetsing van de geselecteerde monoklonale antilichamen (Hoofdstuk 4). Deze optimalisatie werd vervolgens toegepast op de selectie van recombinante antilichamen, die gericht zijn tegen het tomatenbronsvlekkenvirus (TSWV, Hoofdstuk 5). Dat een recombinante antistof een handig stuk gereedschap vormt dat gemakkelijk gemanipuleerd kan worden werd aangetoond door middel van de productie als fusie-eiwit met het enzym alkalische fosfatase (Hoofdstuk 3 en Hoofdstuk 4) en als een fluorescerend eiwit (Hoofdstuk 6).

Curriculum vitae

Op 25 Juli 1963, zag Remko Albert Griep voor de eerste maal het licht in het pittoreske dorp Barsingerhorn. Na de lagere school doorlopen te hebben in Barsingerhorn, begon hij na de MAVO en de HAVO aan zijn voorbereiding op het wetenschappelijk onderwijs in Schagen (Rijks Scholen Gemeenschap). In 1983 behaalde hij zijn VWO diploma, en begon in hetzelfde jaar aan een studie Informatica aan de Technische Universiteit Delft. Helaas konden de levenloze computers hem niet lang boeien en begon hij in 1984 aan de studie Biologie aan de Rijks Universiteit te Utrecht. Het propaedeusediploma volgde een jaar later en hij besloot zich te gaan toeleggen op de afstudeeroriëntatie Chemische Biologie, met afstudeervakken bij de vakgroepen Klinische Immunologie (Prof. dr. R.E. Ballieux) begeleid door Pieter Koolwijk en Moleculaire Celbiologie (Prof. dr. H.O. Voorma) begeleid door Han van Heugten. Hij studeerde af in 1990 en begon als Assistent in Opleiding bij het Laboratorium voor Monoklonale Antistoffen op een vierjarig project dat gefinancierd werd door de programmacommissie landbouw en biotechnologie (PcLB). Het werk uit die periode betrof het maken van monoklonale antistoffen tegen diverse belangrijke plantenpathogenen door middel van recombinant-DNA technologie. Dit werk kreeg in 1994 een vervolg door financiering door de Europese Unie (EEG-AIR3) en de wetenschappelijk relevante resultaten staan beschreven in dit proefschrift.

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R.A. Griep, C. Van Twisk, J.M. van der Wolf and A.Schots. Fluobodies: Green fluorescent single-chain Fv antibodies. (Submitted)

R.A. Griep, C. van Twisk, R. Kormelink, M. Prins, R.J. Kerschbaumer, R. Goldbach and A. Schots. Application of Phage Display in Selecting Tomato Spotted Wilt Virus-specific Single-chain Fvs for Sensitive Diagnosis in ELISA. (Submitted).

A.J.W.G. Visser, R.A. Griep, M. Hink, A. Van Hoek and A. Schots. Translational and rotational diffusion of green fluorescent protein alone and fused with a single-chain Fv protein. (Submitted).

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